MAY 22 1922

Medical Lib.

THE JOURNAL

OF

GENERAL PHYSIOLOGY

EDITORS

JACQUES LOEB W. J. V. OSTERHOUT

VOLUME IV, NO. 5 MAY 20, 1922

PUBLISHED BIMONTHLY

AT MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE, MD.

BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

COPYRIGHT, 1922, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

Entered as second-class matter November 25, 1918, at the Post Office at Baltimore, Md., under the Act of March 3, 1879.

Made in the United States of America

PUBLICATIONS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

THE JOURNAL OF EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

The Journal of Experimental Medicine is designed to cover the field of experimental medicine. It is issued monthly, two volumes of over 600 pages each appearing in a year. The subscription price per year (two volumes) is $5.00, payable in advance. Single copies cost 75 cents. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of Experimental Medicine*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

EDITED BY

STANLEY R. BENEDICT, New York, N. Y. LAFAYETTE B. MENDEL, New Haven, Conn.
H. D. DAKIN, Scarborough, N. Y. DONALD D. VAN SLYKE, New York, N. Y.

The Journal of Biological Chemistry is designed for the prompt publication of original investigations of a chemical nature in the biological sciences. It is issued monthly. Each volume consists of as many numbers as are required to make a total of between 500 and 600 pages. The subscription price is $3.00 per volume to subscribers in the United States and Canada, payable in advance; $3.25 to those in other countries. The number of volumes issued yearly averages four. The price of single copies varies; it is given on the back cover of each number. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of Biological Chemistry*, Mount Royal and Guilford Aves, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

THE JOURNAL OF GENERAL PHYSIOLOGY

EDITED BY

JACQUES LOEB, New York, N. Y. W. J. V. OSTERHOUT, Cambridge, Mass.

The Journal of General Physiology is devoted to the explanation of life phenomena on the basis of the physical and chemical constitution of living matter. Information regarding contributions and subscriptions is given in full on the back cover.

STUDIES FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

The published results of investigations conducted in the Institute, or under its grants, are republished at irregular intervals in volumes designated *Studies from The Rockefeller Institute for Medical Research*. The subscription price is $2.00 per volume, payable in advance. A special rate of $1.00 per volume is made to those who subscribe to *The Journal of Experimental Medicine*, *The Journal of Biological Chemistry*, or *The Journal of General Physiology*. Remittances should be made by draft or check on New York, or by postal money order, payable to The Rockefeller Institute for Medical Research, Avenue A and 66th Street, New York, N. Y.

SEMIANNUAL LIST

The Institute publishes a *Semiannual List* of all papers by members of the staff and those working under grants from the Institute, stating the title and place of publication of these reports. The *Semiannual List* will be sent regularly upon application.

DOES THE KINETICS OF TRYPSIN DIGESTION DEPEND ON THE FORMATION OF A COMPOUND BETWEEN ENZYME AND SUBSTRATE.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 13, 1922.)

Trypsin, in common with many other enzymes, possesses the peculiarity that the rate of formation of the products of hydrolysis does not increase in proportion[1] to the substrate concentration, as is demanded by the law of mass action, but increases more slowly than the substrate concentration and eventually becomes nearly independent of it.[2] It is necessary to assume, therefore, either that the mass law in its simple form does not hold or that the concentration in grams per liter does not represent the "active" concentration of substrate. A very similar phenomenon is quite common in ordinary catalytic reactions. In this case it has usually been assumed that the mass law is valid but that the concentration to be used in the calculation is not the total concentration of the substance taken but that of some particular molecular species which is formed from this substance in solution. In the case of acid hydrolysis, for instance, the action is assumed to be equal to the concentration, not of the acid itself, but of the hydrogen ions. In this case the assumption is capable of verification since the concentration of hydrogen ions may be determined by several independent methods which give approximately the same results, all verifying the assumption. If this were not the case, the kinetics of acid hydrolysis would be more difficult to interpret than the kinetics of enzyme reactions. In the case of enzymes, however, it has usually been assumed, following the

[1] Bayliss, W. M., *Arch. Sc. Biol.*, 1904, xi, suppl., 261.

[2] In the case of alkali or acid hydrolysis this is not true. The rate of digestion in the absence of enzyme is proportional to the gelatin concentration. See Northrop, J. H., *J. Gen. Physiol.*, 1921, iii, 715.

suggestions of Henri[3] and of Brown[4] that the law of mass action in its simple form does not hold but that the velocity of the reaction depends upon the decomposition of a compound between the enzyme and substrate. There is a large amount of evidence that a compound is first formed in many chemical reactions and it has even been stated (Kekule) that no reaction can take place without an addition compound first being formed between the reacting substances. It is quite probable that such a compound is formed in the case of enzyme reactions. The question is whether a sufficient amount of the compound is present at any time to make the kinetics of the reaction depend on the concentration of the compound rather than on the concentration of the reacting substances. Henri,[3] and Michaelis and Menten[5] have attempted to explain the kinetics of invertase hydrolysis by the assumption that the enzyme and substrate combine, according to the law of mass action, to form a compound which subsequently decomposes, liberating the free enzyme and the products of the reaction.[6] It is also assumed that the velocity of hydrolysis depends on the concentration of this compound. It was pointed out by the writer,[7] that if the velocity of hydrolysis depended on the amount of compound formed, then the concentration of substrate required to give the maximum velocity of hydrolysis (*i.e.* to "saturate" the enzyme) should increase with increasing concentrations of enzyme, since it will obviously require more substrate to saturate 100 units of enzyme than it will require to saturate 1 unit. The experiments did not confirm the expectation. It was found that the relative velocity of hydrolysis of different substrate concentrations is always the same, within the experimental error, no matter what enzyme concentration is used (provided the same amount is used with each substrate concentration). It was stated in the article referred to that this was contradictory to the assumption that there was a com-

[3] Henri, V., *Compt. rend. Acad.*, 1902, xxxcv, 916; *Z. physik. Chem.*, 1905, li, 19.

[4] Brown, A. J., *J. Chem. Soc.*, 1902, lxxxi, 373.

[5] Michaelis, L., and Menten, M., *Biochem. Z.*, 1913, xlix, 333.

[6] It has been shown by Simons in Nelson's laboratory that the method used by Michaelis to measure the initial velocity gives values which cannot be used over the entire course of the reaction.

[7] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 595.

pound formed between the enzyme and substrate, since if this were true a greater amount of substrate would be required to saturate a greater amount of enzyme. The experiment, however, is not conclusive, since if it is assumed, as was done by Michaelis and Menten, that the amount of substrate combined with the enzyme is negligibly small, then the difference in concentration of substrate necessary to saturate different amounts of enzyme would be entirely too small to detect experimentally. As far as the relation between the rate of hydrolysis and the concentration of enzyme or substrate is concerned, therefore, the facts may be accounted for by the assumption of an intermediate compound.

It has been shown in a preceding paper[8] that the inhibiting action of the products of the reaction on the trypsin is in quantitative agreement with the assumption that the enzyme and the inhibiting substance combine to form a compound which is inactive and that the rate of hydrolysis is proportional to the concentration of *uncombined* trypsin. It has also been shown that the same assumption will account quantitatively for the protective action of the inhibiting substances when the spontaneous inactivation of the enzyme is followed. The fact that the inhibiting substance protects the enzyme from decomposition is strong evidence that the inhibiting substance combines with the enzyme. In the presence of the substrate, however, the enzyme becomes inactivated at the same rate as the "pure" enzyme[8] (see Ringer).[9] These facts render it unlikely that the enzyme is combined with the substrate. The present paper contains the results of experiments planned to determine whether or not the action of the enzyme with different concentrations of substrate and of inhibiting substances can be accounted for on the assumption of a compound between the enzyme and substrate. The observed facts cannot be accounted for on the basis of the formation of a compound between enzyme and substrate, if it be assumed that this compound is governed by the law of mass action.

[8] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 266.
[9] Ringer, W. E., *Z. physiol. Chem.*, 1921, cvi, 107.

Experimental Methods.

The rate of hydrolysis was followed by means of the change in conductivity of the solution as already described.[8] The experiments were all conducted at a pH of 6.0.

Trypsin.—The trypsin was a sample of Fairchild's trypsin and was purified for use by dialysis under pressure.

Cooper's gelatin was used and was rendered ash-free by washing at the isoelectric point as described by Loeb.[10] The inhibiting solution was made by allowing trypsin to completely digest gelatin and then concentrating the solution *in vacuo*.

Method of Measuring the Rate of Hydrolysis.—In order to obtain a correct measure of the rate of hydrolysis it is necessary to compare the reactions at the same stage. The rate of digestion decreases rapidly with the progress of digestion for two reasons: first, the concentration of substrate is decreasing; second, the concentration of active enzyme is decreasing owing to the inhibiting action of the products of digestion. If the reactions are compared at a point of equal percentage hydrolysis, the change in substrate concentration is corrected for but the change in enzyme concentration will be very different. The small amount of enzyme will be inhibited to a larger extent than the large amount. If the reactions are compared after equal times, both conditions are varied. If, however, the time to cause a very small amount of hydrolysis is taken, the change in substrate concentration may be considered negligible and the effect on the enzyme will be small and nearly the same in both cases. This method, therefore, gives the most significant value.

The result of an experiment with 1 and 5 per cent gelatin and 1 and 10 units of trypsin is shown in Figs. 1, 2, and 3, in which the increase in specific conductivity of the solution has been plotted against the time in hours. Table I gives the time required to cause an equal percentage of the total change in the two gelatin concentrations with the different enzyme concentrations. The table shows that the time required for the hydrolysis to be completed to any given percentage in the two solutions, is not the same (as would be predicted by the monomolecular formula), but is very much greater for the 5 than for

[10] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 237.

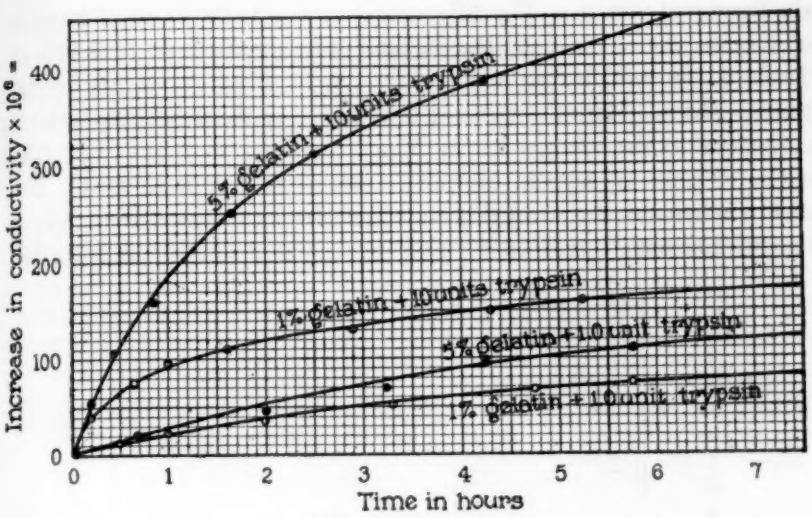


FIG. 1. Rate of digestion of 5 per cent and 1 per cent gelatin with 1 unit and 10 units trypsin as followed by the increase in conductivity.

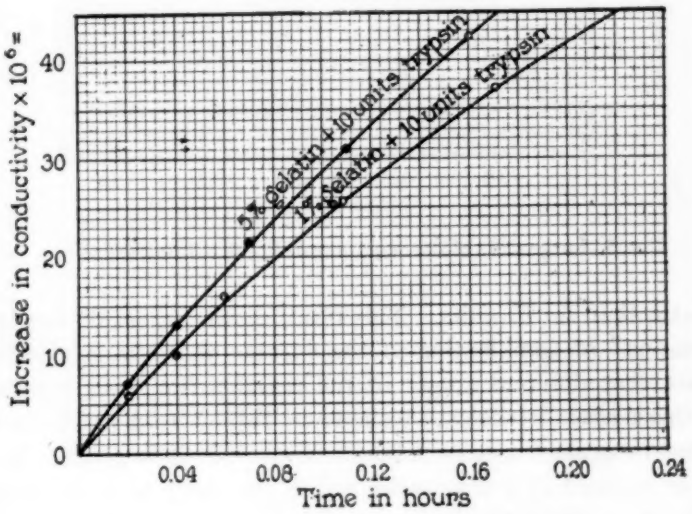


FIG. 2. Large scale of the beginning of Fig. 1. 10 units trypsin.

the 1 per cent gelatin. The difference is greater with the small amount of trypsin than it is with the larger. The same result is shown in Table II in which the change in conductivity after an equal time is given. Here the ratio of the change in the 1 per cent gelatin compared to the change in the 5 per cent gelatin is much smaller when 10 units of trypsin are used than when 1 unit is used. The results when calculated in this way then seem to show that 1 unit of trypsin becomes "saturated" with gelatin at a lower concentration

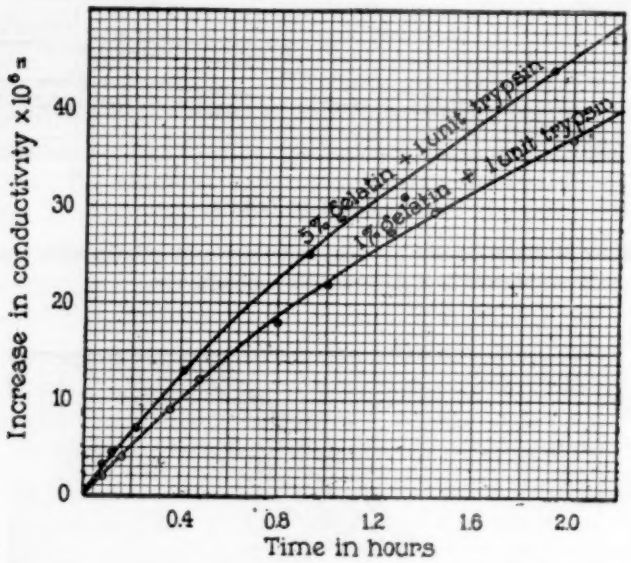


FIG. 3. Large scale of the beginning of Fig. 1. 1 unit trypsin.

of gelatin than do 10 units. This result, however, is not due to the "saturation" or combination of the enzyme with the gelatin but to the fact that different stages of the reaction are being compared. That this is actually the case is shown in Table III, in which the time required to cause the same amount of hydrolysis is given. In this case the amount of products formed is the same in both solutions (*i.e.* the stage of the reaction compared is the same) and, as the table shows, the relative velocity of hydrolysis of the 5 per cent gelatin compared to the 1 per cent gelatin is independent of the amount of

trypsin used. The table also shows that this ratio is constant if the first part of the curve is used but later decreases (*i.e.*, the 5 per cent gelatin is hydrolyzed relatively more and more rapidly), as would be expected, since after any appreciable amount of the gelatin is hydro-

TABLE I.

Time Required for Equal Percentage Hydrolysis of 1 and 5 Per Cent Gelatin Solutions When Compared with (a) 1 Unit of Trypsin, and (b) 10 Units of Trypsin.

Complete hydrolysis of 1 per cent gelatin = increase in conductivity of 0.5 × 10^{-3} reciprocal ohms.

Hydrolysis.	Actual change in conductivity.	1 per cent gelatin hydrolyzed with		Actual change in conductivity.	5 per cent gelatin hydrolyzed with		Relative rate of hydrolysis of 1 per cent gelatin compared to 5 per cent gelatin.	
		1 unit trypsin. (*a*)	10 units trypsin. (*b*)		1 unit trypsin. (*c*)	10 units trypsin. (*d*)	1 unit trypsin. $\frac{c}{a}$	10 units trypsin. $\frac{d}{b}$
per cent	*reciprocal ohms* × 10^3	*hrs.*	*hrs.*	*reciprocal ohms* × 10^3	*hrs.*	*hrs.*		
1	5	0.20	0.018	25	0.90	0.085	4.5	4.7
2	10	0.40	0.037	50	2.24	0.17	5.6	4.6
5	25	1.16	0.104	125	7.50	0.57	6.5	5.5

TABLE II.

Change in Conductivity after Equal Time Intervals.

Time elapsed.	1 per cent gelatin +		5 per cent gelatin +		Ratio, change in 1 per cent gelatin / change in 5 per cent gelatin tested with	
	1 unit trypsin.	10 units trypsin.	1 unit trypsin.	10 units trypsin.	1 unit trypsin.	10 units trypsin.
hrs.	*reciprocal ohms* × 10^3	*reciprocal ohms* × 10^3	*reciprocal ohms* × 10^3	*reciprocal ohms* × 10^3		
0.10	2.5	24	3.5	29	0.7	0.8
0.50	12.5	70	15	115	0.8	0.6
1.00	22	95	28	180	0.8	0.5
3.00	52	135	67	335	0.8	0.4

lyzed the two concentrations are no longer as 5:1 but as 5-a:1-a. As soon as a becomes appreciably large compared to 1, the ratio will evidently increase, as a increases. The ratio of the time required to cause a given change will therefore decrease. This method may,

therefore, be used to determine the relative rate of digestion of different gelatin concentrations provided the change used as end-point is so small that the gelatin concentration can be assumed to remain constant during the course of the experiment.

TABLE III.

Time Required to Cause an Equal Change in the Conductivity of 1 and 5 Per Cent Gelatin Solutions with 1 Unit of Trypsin and 10 Units of Trypsin.

Increase in conductivity.	1 per cent gelatin +		5 per cent gelatin +		Ratio, time for change in 5 per cent gelatin / time for equal change in 1 per cent gelatin with	
	1 unit trypsin.	10 units trypsin.	1 unit trypsin.	10 units trypsin.	1 unit trypsin.	10 units trypsin.
reciprocal ohms × 10^6	*hrs.*	*hrs.*	*hrs.*	*hrs.*		
5	0.19	0.018	0.15	0.014	0.79	0.78
20	0.87	0.08	0.69	0.066	0.79	0.805
50	2.85	0.27	2.05	0.19	0.72	0.71
75	5.75	0.60	3.50	0.30	0.61	0.50

Influence of the Viscosity of the Solution.

It has been suggested that the anomalous results obtained by increasing the concentration of substrate are due to the increased viscosity of the solution. That this is not the cause of the retardation in the present experiments is shown in Table IV which gives the results of an experiment performed with the same gelatin solution which had been kept at 25°C. for varying lengths of time. The viscosity increases slowly under these conditions. As the table shows, the gelatin digests at the same rate whether it has a viscosity of 2.5 times that of water or of 11 times that of water. The physical properties of the gelatin solution evidently have little or no effect on the rate at which it digests.

Influence of the Substrate Concentration.

The results of a series of experiments with varying enzyme and gelatin concentrations are given in Table V. The hydrolysis was followed by the change in conductivity and velocity is taken as the reciprocal of the time required to cause an increase of conductivity

TABLE IV.

Viscosity and Rate of Digestion.

2 per cent gelatin, pH 6.0, specific conductivity 1×10^{-3} (adjusted with NaCl) was heated to 50°C. and cooled rapidly to 25°C. Viscosity was determined at intervals at 25°C. and rate of digestion determined by adding 1 cc. of trypsin to 25 cc. gelatin and following change in conductivity. Increase in formol titration after 1 hr. was also determined.

Viscosity (H_2O = 1.0).		Time to change 10 points.	Formol per 5 cc. after 1 hr. (N/50 NaOH).
At beginning.	After digestion.		
		hrs. × 10^2	*cc.*
2.45			
2.90	1.4	70	2.90
		68	2.90
3.8	1.55	70	2.90
3.9	1.5	69	2.95
4.6	1.6	64	2.95
		76	2.97
7.3	1.7	80	2.95
		80(?)	2.90
		62	
11.3	1.8	70	2.95

TABLE V.

Comparison of Observed and Calculated Results with Varying Gelatin and Trypsin Concentrations.

Concentration of gelatin, *S*.	Rate of hydrolysis $\left(= \frac{100}{T \text{ hrs. to change 5 points in conductivity}}\right) = C$ with enzyme concentrations (*E*).					
	E = 30.0		*E* = 5.0.		*E* = 3.0.	
	Observed.	Calculated.*	Observed.	Calculated.*	Observed.	Calculated.*
per cent						
6.0	27.3	27.7	4.9	4.6	2.7	2.7
3.0	28.4	25.7	4.6	4.3	2.4	2.6
1.5	25.6	22.5	4.0	3.7	2.2	2.2
0.75	17.5	18.0	3.3	3.0	1.8	1.8
0.38	13.1	12.0	2.4	2.0	1.3	1.3

* Calculated from formula $C = \frac{E\,S}{K' + S}$, $K' = 0.5$. The values for *E* are given at the head of the table.

equivalent to 5 points on the bridge. The table shows that the velocity of hydrolysis increases much more slowly than the substrate concentration and becomes practically independent of it in concentrations of more than 3 per cent.[1] The calculated figures were obtained by assuming that the trypsin and gelatin combined according to the reaction

$$\text{trypsin} + \text{gelatin} \rightleftharpoons \text{trypsin} - \text{gelatin}$$

and that the rate of hydrolysis was proportional to the concentration of the *trypsin-gelatin compound.*

Applying the law of mass action to this equilibrium we would have

$$\frac{(E - C) \cdot aS}{C} = KV$$

or

$$C = \frac{ES}{K' + S}$$

in which E equals total amount of trypsin; C, combined trypsin (= combined gelatin); S, amount of gelatin; a, a proportionality factor to change the units of concentration to those of rate of hydrolysis K, the equilibrium constant; V, the volume of solution; and K', a new constant equal to

$$\frac{KV}{a}$$

It will be seen that if C is considered negligibly small, compared to E, as well as to S, the equation reduces to Rate $= kC = KES$ which is the ordinary form of the law of mass action.

Evaluation of the Constants.

Since it is assumed that the rate of hydrolysis is proportional to the amount of the gelatin-trypsin compound (C) present, C is the observed velocity.

E, the total amount of trypsin, cannot be determined directly but is taken as a value slightly larger than the maximum value obtained for C, when the substrate concentration is such that the hydrolysis proceeds at the maximum rate. According to the hypothesis, this maximum value is due to the fact that practically all the enzyme is combined, and since C (the rate of hydrolysis) is a measure of the amount combined, E must be very slightly larger. E is, therefore, an arbitrary constant. After a value for E has been determined for a given amount of trypsin solution, the value to be used with any other amount of trypsin will be propor-

tional to the relative amount of trypsin taken, *i.e.*, if E_0 is the value used when 1 cc. of trypsin is taken then nE_0 will be the value for n cc.

K' is determined by substituting the values of C, E, and S and solving for K'. It is, therefore, a second arbitrary constant.

Table V shows that the calculated and observed values are concordant. It also shows that the relative rate of hydrolysis of any two gelatin concentrations with any one trypsin concentration is independent of the value of this trypsin concentration.

The results when the enzyme or substrate concentrations are varied, therefore, agree with those predicted by the assumption that the enzyme and gelatin combine to form a compound and that the rate of hydrolysis is proportional to the concentration of this compound.

The equation which was used to calculate these values, however, contains two arbitrary constants and is of a form to fit any curve which at first shows direct proportionality and then approaches a maximum value. It is not surprising, therefore, that the calculated and observed results agree. The test of an equation of this type is to see whether or not it will fit the results of an experiment other than the one from which the values of the constants was originally obtained. As will be seen below, this is not the case; the equation breaks down when the experiment is performed in the presence of inhibiting substances.

Influence of the Gelatin Concentration on the Retarding Effect of Inhibiting Substances.

It was found[8] in studying the influence of the inhibiting substance on the rate of digestion that the experiments agreed with the assumption that the enzyme and inhibitor combined to form a compound that was inactive and that the rate of hydrolysis was proportional to the concentration of *free enzyme*. It was also found that there was direct evidence that the inhibitor affects the enzyme and not the substrate. The experiment summarized in Table V, however, if taken alone, shows that the influence of the substrate concentration agrees with the assumption that the rate of hydrolysis is proportional to the amount of *enzyme combined* with the substrate. It is evident that both assumptions cannot be correct.

The direct experimental evidence shows conclusively that the enzyme and the inhibiting substances are combined to form a highly dissociated compound, even though the agreement of the experiments with the results calculated from the law of mass action be considered to be accidental. If the substrate is also combined with the enzyme it should be possible, by increasing the substrate concentration sufficiently, to cause all the enzyme to combine with the substrate. In other words, the higher the substrate concentration the less should be the effect of the inhibiting substance. This may be seen from the following equation:

$$\text{trypsin} \begin{cases} + \text{gelatin} \rightleftarrows \text{trypsin-gelatin} \\ \longrightarrow \\ + \text{inhibitor} \rightleftarrows \text{trypsin-inhibitor} \end{cases}$$

Increasing the concentration of gelatin will cause the equilibrium to be shifted in the direction of the large arrow; *i.e.*; it will cause the amount of trypsin combined with the inhibitor to become less. This will be true even though the equilibrium is not one which follows the law of mass action, as long as the equilibrium is reversible and the trypsin-inhibitor compound widely dissociated. It has already been found that the trypsin-inhibitor compound is readily dissociated irrespective of any assumption as to the nature of the compound. Table VI is a summary of experiments made with constant amounts of trypsin and inhibiting substance and increasing concentrations of gelatin. The figures are the averages of four to six determinations. The table shows that the retardation due to the inhibiting substance is independent of the gelatin concentration. In order to account for this result, if the velocity of hydrolysis depends on a trypsin-gelatin compound, it is necessary to assume that the trypsin-inhibitor compound is only very slightly dissociated, and that the inhibiting substance and trypsin are present in about the same concentration. The figures under Calculated I were obtained by means of the law of mass action based on these assumptions. They approximate the experimental values and agree with the experimental result that the percentage retardation is independent of the gelatin concentration. If it be assumed, as was done by Michaelis and Menten, that the inhibiting substance is present in very much higher concentration than the enzyme, the figures given under Calculated II are obtained. They are evidently incompatible with the experiment.

TABLE VI.

Influence of the Gelatin Concentration on the Retardation Caused by the Presence of a Constant Amount of Inhibiting Substances.

Concentration of gelatin, S.	C = rate of hydrolysis in					Ratio of rate of hydrolysis of $\frac{\text{control}}{\text{inhibitor}}$ Observed.
	Control solution.		Solution containing inhibitor.			
	Observed.	Calculated.*	Observed.	Calculated (I).*	Calculated (II).†	
per cent						
8	10.0	9.5	7.1	6.6	[7.1]	1.43
4	10.0	9.0	7.0	6.3	5.4	1.43
2	9.6	8.1	6.8	5.7	3.7	1.41
1	7.3	6.8	5.0	4.7	2.3	1.46

* Calculated by equation $C = \frac{S(E-I)}{K+S}$. $E = 10.1$, $I = 3.0$ (units inhibitor), $K = 0.5$ (derived by assuming that inhibitor-enzyme compound is very little dissociated).

† Calculated by equation $C = \frac{E\,S}{K''+S} = 10.1$, $K'' = 3.4$, derived by assuming that the amount of inhibitor combined with the enzyme is negligible compared to total amount of inhibitor, and that inhibitor enzyme compound is widely dissociated.

This equation is derived as follows: Let S = total substrate concentration, E total enzyme concentration, C enzyme combined with substrate, J enzyme combined with inhibitor, and I total inhibitor.

Then if the amount of substrate combined with the enzyme is negligibly small compared to the total amount of substrate and if the same is true of the inhibitor, the mass action expressions for the two equilibria are

$$S(E-C-J) = K\,C\ (1) \qquad I(E-C-J) = k\,J\ (2)$$

$$J = \frac{E\,S - K\,C - S\,C}{S}$$

Substituting this value of J in (2) and simplifying $C = \frac{E\,S}{\frac{I\,K}{k}+K+S}$ in which K = equilibrium constant of substrate-enzyme equilibrium, and k = equilibrium constant for inhibitor-enzyme compound. Since in this experiment I, K, and k are all constant they may be combined to a new constant K'' and $C = \frac{E\,S}{K''+S}$.

Effect of Varying the Amount of Trypsin or Inhibiting Substance.

It has been shown above that in order to account for the fact that the percentage retardation is independent of the substrate concentration, it is necessary to assume that the inhibitor-trypsin compound is only slightly dissociated. This assumption, as has already been pointed out, is contradicted by the experiments in which the amount of trypsin or inhibitor is varied. This is shown in Tables VII and VIII. In these tables the results under Calculated I were obtained from the equation used to calculate the results in Table VI, and which is derived by means of the assumption that the inhibitor-trypsin compound is only slightly dissociated. The table shows that the formula will not serve even as a first approximation in spite of the fact that it contains three arbitrary constants. The figures given under Calculated II were obtained by aid of the assumption that the enzyme-inhibitor compound is widely dissociated and that the rate of hydrolysis is proportional to the free enzyme.[11] They agree well with the experimental values.

These experiments show that the results obtained when the gelatin, inhibitor, and trypsin concentrations are all varied cannot be accounted for on the assumption that the trypsin becomes saturated with substrate. They seem to be conclusive even though it is assumed that the equilibria are not governed by the law of mass action, since in order to explain one set of experiments (gelatin constant, trypsin or inhibitor varied) it is necessary to suppose that the trypsin-inhibitor compound is widely dissociated while in the other set of experiments (trypsin and inhibitor constant, gelatin varied) it is necessary to suppose that the same compound is very slightly dissociated. This is true irrespective of the quantitative law that is assumed to govern the equilibrium. There is much more direct experimental evidence in favor of the trypsin-inhibitor compound than of the trypsin-gelatin compound.

There is no doubt on the other hand that the rate of hydrolysis does not increase in proportion to the gelatin concentration as expressed in grams per liter. If it is assumed then that the reaction is

[11] For the derivation of this equation see Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 230.

TABLE VII.

Effect of Increasing the Concentration of Inhibitor. 2 Per Cent Gelatin.

Inhibiting solution.	Rate of hydrolysis = C.		
	Observed.	Calculated (I).*	Calculated (II).†
cc.			
0.0	2.3	2.3	[2.3]
0.125	1.9	[1.9]	1.81
0.25	1.56	1.5	1.45
0.50	1.10	0.72	1.00
1.0	0.65	<0.10	0.58
2.0	0.33	<0.01	0.32

* Calculated from $C = \frac{S\,(E - I \text{ cc.})}{K + S}$; *i.e.*, enzyme-inhibitor compound very slightly dissociated; velocity proportional to *combined enzyme* (C). $E = 2.9$, $S = 2, K = 0.5, I = 4.0$ units inhibitor per cc. of solution.

† Calculated from $Q = \sqrt{\left(\frac{d + K - E}{2}\right)^2 + K\,E} - \frac{d + K - E}{2}$

Assumptions: (1) enzyme-inhibitor compound widely dissociated. (2) Rate of hydrolysis proportional to *free* enzyme (Q). $K = 2.8$, $E = 2.3$, d = cc. inhibiting solution $\times$ 10.

TABLE VIII.

Effect of Varying Enzyme Concentration with Constant Gelatin and Constant Inhibitor Concentrations.

Gelatin 2 per cent. 25 cc. + 1 cc. inhibiting solution + noted cc. trypsin solution.

Trypsin.	E_1	E_2	Rate of hydrolysis.		
			Observed.	Calculated.*	Calculated.†
cc.					
1.1	13.0	11.0	7.4	[7.4]	[7.4]
0.55	6.6	5.5	3.2	2.3	3.0
0.30	3.6	3.0	1.4	<0.01	1.35
0.15	1.8	1.5	0.80	<0.01	0.70

* Calculated by formula $C = \frac{S\,(E_1 - I)}{K + S}$, $S = 2$, $I = 3.7$, $K = 0.5$, E_1 as in table. Assumptions: (1) enzyme-inhibitor compound slightly dissociated; (2) velocity proportional to combined enzyme.

† Calculated by same formula as (II), Table VII. $d = 5.0$. E_2 as in table above.

governed by the law of mass action and that the velocity of hydrolysis is really proportional to the concentration of free trypsin and protein, it is necessary to suppose that the reaction is confined to some particular molecular species present in the protein solution or to introduce a "catalysis" coefficient to express the ratio of actual concentration to "active" concentration as has been done in the case of hydrogen ion by Schreiner.[12] The work of Loeb, Michaelis, Sörensen, Robertson, and others has shown that proteins in solution are ionized so that it would be natural to suppose that the speed of reaction is proportional to the concentration of protein ions instead of to the total concentration of protein. It has been found that in the case of pepsin hydrolysis this accounted for the difficulty both as regards differences in the concentration of protein and the effect of the hydrogen ion concentration of the solution. In the present case, however, the ionic concentration, as measured by the conductivity of the solution, increases more rapidly than the rate of hydrolysis but less rapidly than the concentration so that the anomaly is only partly corrected for.

It has been found by von Euler and Svanberg[13] in the case of invertase that the retardation due to inhibiting substances is independent of the substrate (sugar) concentration so that in the case of this enzyme also the evidence is contradictory to the assumption of a substrate-enzyme compound.

Hydrolysis of Mixtures of Casein and Gelatin.

The rate of hydrolysis of casein solutions increases less rapidly than the concentration of casein, just as in the case of gelatin. This is shown in Table IX. It is evident that increasing the concentration of casein above 4 per cent has little or no effect on the rate of digestion. According to the saturation hypothesis the trypsin must, therefore, be "saturated" with casein when the latter is at a concentration of 4 per cent or more. It is interesting to consider the digestion of a mixture of casein and gelatin from the points of view

[12] Schreiner, E., *Z. anorg. Chem.*, 1921, cxvi, 102.

[13] von Euler, H., and Svanberg, O., *Fermentforschung*, 1921, iv, 142.

of the various possible assumptions.[14] The following possibilities present themselves and may be compared with the experiment shown in Fig. 4 and Table X. The values are the mean of 4 to 6 determinations.

TABLE IX.

Effect of Increasing Casein Concentrations on the Amount of Casein Digested.

pH 8.0. Phosphate buffer. 34°C.

Casein concentration, *per cent*	0.5	1.0	2.0	3.0	5.0
Increase of amino nitrogen per cc. solution after 1 hr., *cc*	0.20	0.33	0.45	0.54	0.55

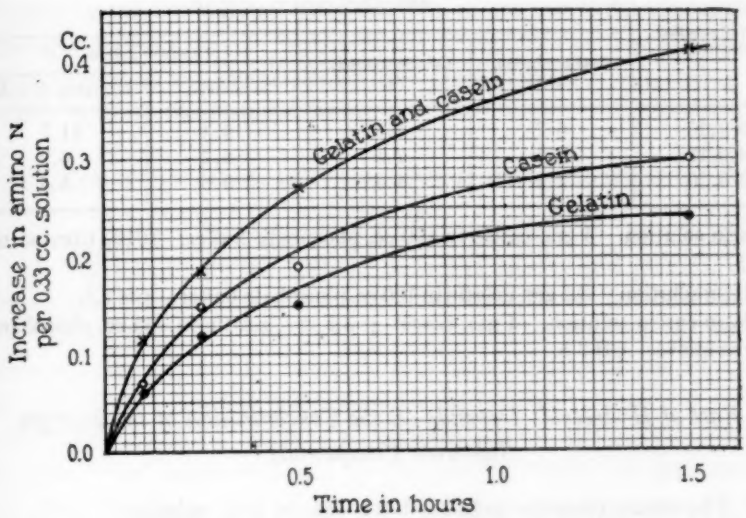


FIG. 4. Digestion curves for 4 per cent casein, 3 per cent gelatin, and a mixture containing both 3 per cent gelatin and 4 per cent casein with the same concentration of trypsin.

[14] This case was considered by Henri and des Bancels (Henri, V., and des Bancels L., *Compt. rend. Soc. biol.*, 1903, lv, 866), who, however, failed to distinguish between the *rate of hydrolysis* and the *amount of hydrolysis*.

TABLE X.

Rate of Hydrolysis of Casein, Gelatin, and a Mixture of Casein and Gelatin.

4 cc. dialyzed trypsin were added to each solution at 34°C. 5 cc. samples were removed after 0.10, 0.25, 0.50, 1.50, and 3.0 hrs. and run into 25 cc. of water containing 10 cc. 0.20 N HCl. 2 cc. of this solution (equivalent to 0.33 cc. of original solution) were analyzed for amino nitrogen by Van Slyke method.

Increase in NH_2 N. (*a*)	Time required to cause an increase of amino nitrogen noted under *a* in		
	Casein solution.	Gelatin solution.	Casein + Gelatin.
cc.	*hrs.*	*hrs.*	*hrs.*
0.1	0.15	0.20	0.09
0.15	0.27	0.40	0.16
0.20	0.48	0.72	0.28

Stage of reaction compared.	Rate of hydrolysis = $\frac{1}{T \text{ hrs.}}$ in			
	Casein solution. (*b*)	Gelatin solution. (*c*)	Casein + gelatin.	
			In mixture.	Separately (*b* + *c*).
0–0.10	6.7	5.0	11.1	11.7
0–0.15	3.7	2.5	6.2	6.2
0–0.20	2.1	1.4	3.6	3.5

Casein solution. 4 gm. casein in 100 cc. phosphate buffer. M/10, titrated to pH 7.5.

Gelatin solution. 3.5 gm. gelatin in 100 cc. phosphate buffer. pH 7.5.

Gelatin-casein solution. 4 gm. casein + 3.5 gm. gelatin in 100 cc. phosphate buffer as above. pH 7.5.

I. Rate of Hydrolysis Depends on the Concentration of the Enzyme Substrate Compound.

(*a*) The same enzyme acts on both casein and gelatin.

In this case the rate of hydrolysis of the mixture must be less than the rate of hydrolysis of the casein alone, since it has already been assumed, in order to account for the rate-concentration curve, that the enzyme is saturated by 4 per cent casein. The addition of gelatin to the system will therefore remove some enzyme from the casein to combine with the gelatin and since the gelatin hydrolyzes more slowly than the casein, the result will be a decrease in the rate of digestion. This is contradicted by the experiment.

(*b*) The casein and gelatin are hydrolyzed by different enzymes acting entirely independently.

The amount digested at any time in the mixture must then be equal to the sum of the amounts digested at the same time when the casein and gelatin are hydrolyzed separately. The same result would be predicted if the rate of hydrolysis depended on the concentration of free enzymes. This is also contradicted by the experiment.

(*c*) The casein and gelatin are acted on by two different enzymes, but the products formed by either enzyme inhibit the action of the other.

The rate of hydrolysis of the mixture, according to this mechanism, will equal the sum of the rates of hydrolysis of the two separate solutions but the amount of hydrolysis at any given time will be slightly less in the mixture than the sum of the two separate solutions. The same result would be predicted if the rate of hydrolysis were proportional to the concentration of free enzymes.

This is the experimental result.

II. Rate of Hydrolysis is Proportional to the Concentration of Free Enzyme.

(*a*) The same enzyme acts on both the casein and gelatin. This assumption predicts that the rate of hydrolysis of the mixture will be equal to the sum of the rates of the two solutions but the amount of hydrolysis of the mixture will be less than the sum of the two separate solutions. This is the experimental result.

We are, therefore, bound to conclude either, first, that the rate of hydrolysis is proportional to the concentration of free enzyme (*i.e.* that the amount combined is negligibly small), or second, that there are two enzymes at work, each of which is inhibited by the products of hydrolysis formed by the other. This latter assumption is gratuitous unless some independent evidence can be found for the existence of two such enzymes. Many experiments were made from this point of view but no evidence could be found for the existence of two enzymes. The ratio of the rate of hydrolysis of gelatin and casein was always the same within the experimental error of about 1 per cent, no matter how the trypsin preparation was treated.

Rate of Hydrolysis as Measured Directly by the Disappearance of the Substrate.

It has been shown above that the rate of formation of the products of hydrolysis of gelatin or casein by trypsin does not increase in proportion to the concentration of substrate but increases much more slowly and becomes independent of the substrate concentration when the latter is more than 2 or 3 per cent. It was also shown that this peculiarity could not be accounted for by assuming the existence of an intermediate compound between the enzyme and substrate nor by the assumption that the hydrolysis was proportional to the ionized protein. In these experiments as in most experiments with enzymes the hydrolysis was followed by determining the amount of the products formed and assuming that the amount of substrate remaining is the difference between the amount of products found at any time and the total amount that can be formed under the most favorable conditions. It is well known that trypsin digestion consists of a series of consecutive reactions since a number of products may be isolated from a digestion mixture which can still be acted on by the enzyme. It seemed possible therefore that the peculiar results discussed above were due to the fact that the increase in the products of reaction does not correctly represent the decrease in the substrate concentration. It is the change in concentration of the latter value that is predicted by the law of mass action. An experiment was therefore performed in which the digestion was followed by determining the increase in amino nitrogen and also the *decrease* in unchanged casein. The results are given in tables XI and XII. The tables show that the two methods give entirely different results. As measured by the increase in amino nitrogen the rate of hydrolysis is practically independent of the casein concentration, whereas when the change in the casein concentration is measured directly the rate of digestion is very nearly proportional to the concentration of casein as demanded by the law of mass action. The constant calculated from the monomolecular formula still shows a drop with increasing hydrolysis. This is more marked in the concentrated than in the dilute solution and is the result expected owing to the inhibiting action of the products of hydrolysis. When the rate of hydrolysis is de-

TABLE XI.

Effect of Increasing Concentration of Casein on the Rate of Hydrolysis as Measured by the Increase in Amino Nitrogen.

Casein dissolved in a mixture of M/20 Na_2HPO_4, H_3BO_3, and Na_3 citrate of pH 8.0. 100 cc. of this solution + 1 cc. dialyzed trypsin at 34°C. for time noted. Amino nitrogen determined by Van Slyke method on 0.6 cc. of solution as noted.

Increase in amino nitrogen per 0.6 cc. solution containing noted concentration of casein.

After hrs. at 30°C.	Gm. of casein per 100 cc.		
	2 gm.	4 gm.	6 gm.
hrs.	*cc.*	*cc.*	*cc.*
0.5	0.20	0.20	0.24
1.0	0.25	0.26	0.25
2.0	0.30	0.38	0.42

The figures are the mean of 4 to 6 determinations. The average deviation of the mean is about 5 per cent.

TABLE XII.

Effect of Increasing Concentration of Casein on Rate of Digestion as Measured by the Decrease in Undigested Casein.

Casein dissolved in M/20 phosphate, borate, citrate buffer pH 8.0. 100 cc. solution + 1 cc. dialyzed trypsin placed at 34°C. and 10 cc. samples removed as noted, titrated to pH 4.6, and added to 100 cc. 0.1 N acetate buffer pH 4.6. Precipitate filtered, dried at 100°C., and weighed.

		Hrs. at 34°C.			
		0	0.25 hr.	0.50 hr.	1 hr.
Casein per 10 cc. solution, *gm.*	*a*	0.15	0.08	0.05	0.02
	b	0.31	0.19	0.12	0.08
	c	0.50	0.31	0.26	0.23
Monomolecular constant for solution.	*a*		1.00	0.96	0.87
	b		0.90	0.84	0.60
	c		0.85	0.60	0.34
Time to dissolve 0.02 gm. in solution, *hrs.*	*a*	0.08			
	b	0.045			
	c	0.025			
K = concentration casein × time to dissolve 0.02 gm.	*a*	0.0120			
	b	0.0140			
	c	0.0125			

termined at the same stage of the reaction by interpolation from the time curves, it is found that the rate of hydrolysis increases in direct proportion to the casein concentration. This is shown by the last line of Table XII. The same result is shown by comparing the monomolecular constants at corresponding values of x. This experiment shows that when the substrate concentration is measured directly the reaction proceeds according to the law of mass action both as regards the concentration of enzyme and of substrate and that the only divergence from the simple monomolecular formula is due to the fact that the enzyme concentration also changes during the course of the reaction, owing to the effect of the products of the reaction, an effect which can easily be demonstrated directly. The experiment described was repeated several times with the same result. It was also found that if the rate of hydrolysis of gelatin was followed by means of the change in viscosity (which is roughly proportional to the gelatin concentration), the same result was obtained. This indicates that in the case of gelatin as well, the apparent discrepancy from the law of mass action is due to the fact that the reaction is really a series of consecutive reactions and that the change in concentration of the original substance cannot be determined from the increase in the total products of reaction. It will be seen that this mechanism will account also for the fact that the increase in the products of reaction as plotted against the time is occasionally a straight line. Assume, for instance, that the reaction may be written $A \rightarrow B \rightarrow C$ and that C is what is determined experimentally. The rate of formation of C will evidently be proportional to the concentration of B at any instant, and this in turn will depend on the relation between its rate of formation and of decomposition. It is possible therefore for the rate of formation of C to remain constant, increase or decrease with time, and there is not necessarily any simple relation between the concentration of A and the rate of formation of C.

SUMMARY.

1. The velocity of hydrolysis of gelatin by trypsin increases more slowly than the gelatin concentration and finally becomes nearly independent of the gelatin concentration. The relative velocity of

hydrolysis of any two substrate concentrations is independent of the quantity of enzyme used to make the comparison.

2. The rate of hydrolysis is independent of the viscosity of the solution.

3. The percentage retardation of the rate of hydrolysis by inhibiting substances, is independent of the substrate concentration.

4. There is experimental evidence that the enzyme and inhibiting substance are combined to form a widely dissociated compound.

5. If the substrate were also combined with the enzyme, an increase in the substrate concentration should affect the equilibrium between the enzyme and the inhibiting substance. This is not the case.

6. The rate of digestion of a mixture of casein and gelatin is equal to the sum of the rates of hydrolysis of the two substances alone, as it should be if the rate is proportional to the concentration of free enzyme. This contradicts the saturation hypothesis.

7. If the reaction is followed by determining directly the change in the substrate concentration, it is found that this change agrees with the law of mass action; *i.e.*, the rate of digestion is proportional to the substrate concentration.

Most of the experimental work in this paper was done by Mr. Frank Johnston.

THE INFLUENCE OF X-RAYS ON THE PROPERTIES OF BLOOD.

By RAYMOND G. HUSSEY.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 29, 1922.)

In the course of an experimental investigation concerning the nature of the reaction of the animal organism to x-rays we have made observations which we believe to be of sufficient interest to communicate in the form of a preliminary report. Our experiments were originally designed to throw some light on the question raised by the statement made by some roentgenologists that the characteristic illness frequently observed in individuals after an exposure to massive therapeutic doses of x-rays is due to a state of acidosis. The basis of this statement is apparently an empirical one since there is no rational evidence to support such an idea. We do not interpret the results of the experiments to be reported in this paper as a final answer to this question. We shall discuss this subject in a later paper.

Our procedure has been to study the numerical changes in the white cells of the blood, and the pH and bicarbonate content of the plasma in rabbits following an exposure of these animals to x-rays. Rabbits were chosen as the most convenient of the usual laboratory animals for our first observations on account of the facility with which they can be bled from the heart. We realize, however, that they are not ideal animals for our ultimate purpose. The plasma was obtained from blood drawn under paraffin oil into a tube which contained a sufficient amount of potassium oxalate to prevent coagulation. From 15 to 20 cc. of blood were drawn and the final oxalate concentration was about 0.3 per cent. Immediately after drawing the blood the plasma was separated from the cells by centrifuging. The surface of the oil was covered with low freezing point paraffin before centrifuging to prevent contact with air. We have found it possible to bleed a rabbit four or five times a week without any apparent effect on the

general reaction of the animal, as regards the conditions of our investigation. For the pH determinations we have employed the colorimetric method developed by Cullen,[1] and for determining the bicarbonate content we have employed the procedure of Van Slyke.[2] The pH determinations have been checked electrometrically as have also the standard buffer solutions used in the colorimetric tests.

Preliminary observations showed the range of the pH value of rabbit plasma to be between 7.12 and 7.26, values which are lower than those stated by Hasselbalch and Lundsgaard (1912), 7.33 (electrometric), and Kurijama (1913), 7.4 to 7.5 (colorimetric). However, since the electrometric method employed in obtaining our values is more precise than was the method available at the time the values of Hasselbach and Lundsgaard were determined, we believe our values to be more nearly correct. We found the normal plasma bicarbonate content, expressed in volumes per cent of CO_2, to vary between 26 and 45. It is possible to obtain wide variations in a single rabbit unless careful attention is given to keeping the animal quiet during the process of bleeding. Feeding also plays a rôle in the variations of different samples of plasma but we have found it possible to control this factor by standardizing the amount of food and the time of feeding and the interval of time between the removal of animals from food before the beginning of the experiment.

The rabbits have been exposed to x-radiation from a Coolidge tube, the dosage factors being as follows: spark gap, 3 inches; current, 10 milliamperes; target distance, 6 inches; and time of exposure, 15 minutes. This is a massive dose of radiation of relatively long wave lengths, a large part of which are absorbed by the skin. This dose approaches the maximum amount that can be given without killing the animal and was purposely chosen as a starting point in order to obtain clear cut results. The exposures were made directly on the rabbit's abdomen which had been previously shaved.

EXPERIMENTAL.

1. Four rabbits were bled from the heart for plasma, then two of the animals were exposed to the radiation stated. The other two

[1] Cullen, G. E., *J. Biol. Chem.*, 1922, p. xvii.

[2] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

animals were not x-rayed. One control and one test animal were bled again 24 hours later and the other control and test animals were bled 48 hours after the x-ray exposure. 2 weeks later the test animals were bled again. The observations made on the plasma obtained at these times are given in Table I.

TABLE I.

Rabbit No.	Before radiation.		24 hrs. after radiation.		48 hrs. after radiation.		14 days after radiation.	
	pH	CO_2	pH	CO_2	pH	CO_2	pH	CO_2
		vol. per cent		*vol. per cent*		*vol. per cent*		*vol. per cent*
1 (x-rayed.)	7.16	33.2	7.34	51.0			7.22	51.2
3 "	7.21	35.6			7.35	49.6	7.23	48.6
2 (Normal.)	7.20	35.2	7.15	33.5				
4 "	7.19	38.6			7.14	34.7		

This experiment was repeated with results similar in magnitude to those given.

2. Two rabbits were bled then x-rayed as stated. Blood was obtained from one of them an hour after the x-ray exposure, and from the other 3 hours after. The results of the observations on the plasma obtained are given in Table II.

TABLE II.

Before radiation.		1 hr. after radiation.		3 hrs. after radiation.	
pH	CO_2	pH	CO_2	pH	CO_2
	vol. per cent		*vol. per cent*		*vol. per cent*
7.26	38.5	7.30	48.0		
7.24	38.8			7.35	54.6

In other experiments observations have been made on the plasma at various intervals following exposure to the x-rays and the above results corroborated.

Blood counts have been made on rabbits which were bled as well as x-rayed, and on others which were x-rayed only the changes do not differ significantly. When counts are made at 15 minute intervals the first decrease we have noted on the mononuclear elements occurs

½ hour after the x-ray exposure. After the first hour the interval between counts has been 1 hour and the maximum decrease occurs between 3 and 5 hours, the same period noted for the maximum change in the reaction of the plasma.

Summarized briefly the results of these experiments show that there exists a definite alkali excess in the body of the rabbit following an exposure to the dose of x-rays employed. Since there is an increase in the bicarbonate content of the plasma as well as an increase in the pH (*i.e.* a decrease in the C_{H^+}), then evidently the $\frac{BA}{HA}$ ratio must be disturbed in a manner which defines a state of uncompensated alkali excess.[3] The maximum change in the reaction of the plasma is reached between 3 and 5 hours following exposure to radiation. The pH is observed to reach a normal level before the bicarbonate, which indicates the existence of a period of compensated alkali excess before the animal returns to normal. Evidence is found which significantly associates the point of maximum change in the reaction of the plasma with the maximum change noted in the white cell counts of the blood.

With these facts established we directed our attention to the possibility of producing a state of alkali excess in the rabbit by injecting sodium bicarbonate, and to the study of any chemical and morphological blood changes which might ensue. As a guide to the amount of salt to inject, we availed ourselves of the formula developed by Palmer and Van Slyke,[4] for bicarbonate administration with reference to the effect of a given amount of salt on the reaction of the body fluids. The formula is based on the fact that 1 gm. of $NaHCO_3$ yields 267 cc. of CO_2 measured under standard conditions. If now it is assumed that 0.7 of the body weight is fluid, it is obvious that there are 700 cc. of fluid for each kilo of body weight and 1 gm. of bicarbonate would increase the CO_2 content $\frac{267}{7W}$ volumes per cent. This expression simplifies to $\frac{38}{W}$ where W is the body weight expressed in kilograms. If now we let b = the volume per cent increase in CO_2, we

[3] Van Slyke, D. D., *J. Biol. Chem.*, 1921, xlviii, 1.

[4] Palmer, W. W., and Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxxii, 499.

have the simple equation $b = \frac{38}{W}$. Obviously for g gm. of $NaHCO_3$ the equation becomes $g = \frac{bW}{38}$. As our results show, this equation permits one to calculate with a good degree of accuracy the change to be expected.

Four rabbits were bled and the bicarbonate content and pH of their plasma was determined. Later they were given an intraperitoneal injection of sodium bicarbonate in amounts stated and the reaction of their plasma was again determined after an interval of 1 hour with the results given in Table III.

TABLE III.

Rabbit No.	Weight.	$NaH\ CO_3$ injected.	Before injection.		1 hr. after injection.			Difference between calculated and observed CO_2
			pH	CO_2	pH	CO_2 observed.	CO_2 calculated.	
	kilos	*gm.*		*vol. per cent*		*vol. per cent*	*vol. per cent*	*vol. per cent*
1	2.3	1	7.14	36.2	7.30	67.0	69.2	−2.2
2	2.3	3	7.19	35.6	7.42	88.1	85.2	+2.9
3	2.2	1	7.17	37.6	7.35	52.2	54.9	−2.7
4	2.1	1	7.22	40.6	7.36	55.4	59.9	−4.5

In other experiments similar observations have been made at varying intervals following injections of 1 gm. of bicarbonate. All injections were made into the peritoneal cavity. For periods after 1 hour the observed values are much less than the calculated values. The pH returns to normal in about 3 hours, but the bicarbonate content remains high up to 5 hours.

Observations on the white blood cells of these rabbits, as well as on other rabbits similarly treated show a diminution of white blood cells per cc. and the absolute counts show this to be due largely to the mononuclear forms. The maximum effect is noted 1 to 3 hours after the injection of the salt which time coincides with the period of maximum change in the reaction of the blood. This is true also in the rabbit following exposure to x-rays. Immediately following this maximum decrease there is a continued rise in the number of white blood cells per cc. and a rapid return to normal. It is possible, however, to maintain a low level of the mononuclear cells by repeated

injections of the salt. This is true in the guinea pig too, the only other animal to which we have so far extended these observations. Following an injection of 5 cc. of physiological saline solution we observe no significant change in the blood picture compared with blood counts on normal animals made at similar intervals.

DISCUSSION.

The facts thus presented develop an interesting analogy between the changes associated with x-ray effects and those found following injections of sodium bicarbonate. We do not feel justified at this time in attempting to make a definite interpretation of these results. The analogy, however, seems to be a striking one and it is very suggestive for further study, since it presents a possible opening for investigations concerning the mechanism of x-ray action on the animal body. Furthermore, evidence is found which justifies a doubt that the characteristic cell changes noted following x-ray exposures are due to the direct action of the x-rays. Additional force is given this doubt by the results of further experiments now in progress in which we are observing the effects accompanying the injection of other substances. So far we have used sodium chloride, sodium sulfate, monosodium phosphate, and glucose. Very definite changes are noted in the blood picture, but the data we have to date are not sufficient to permit of any further statement.

CONCLUSION.

When rabbits are exposed to x-radiation as described, there results a definite change in the $\frac{BA}{HA}$ ratio of their plasma. This change is evidently one which defines a state of uncompensated alkali excess.

The time required for a maximum change in the chemical reaction is the same as that required for a maximum change in the decrease of leucocytes.

Sodium bicarbonate injected into the peritoneal cavity is followed by results identical with those observed following exposure to x-rays. The maximum changes occur in a shorter time following bicarbonate injection, but the relation between chemical and morphological changes are the same.

We consider this analogy to be an important one in that it is suggestive of a relationship between the effect of salt and the effect of x-rays.

KINETICS OF THE BIOLUMINESCENT REACTION IN CYPRIDINA. I.

By WILLIAM R. AMBERSON.

(From the Physiological Laboratory, Princeton University, and the Laboratory of Pure Science, Nela Research Laboratories, Cleveland, Ohio.)

(Received for publication, March 21, 1922.)

INTRODUCTION.

Until the present time it has been proven impossible to isolate as pure chemical individuals any of the reactants concerned in the production of light by organisms. The work of Dubois[1] and Harvey has, however, indicated the general nature of such reactions as consisting in the oxidation of a substrate, luciferin, catalyzed by an enzyme, luciferase. Harvey[2] believes that luciferin should be placed among the natural proteoses on the borderland between the proteoses and peptones, whereas luciferase is a protein probably belonging to the albumin group.

Quantitative studies upon such reactions are beset with several difficulties, among the most important of which are (1) absolute lack of information as to the concentration of the reactants present in extracts, and (2) the rather highly labile character of the substrate, luciferin, which readily oxidizes spontaneously without light production in the absence of the enzyme. I have made an attempt, however, to discover something of the time relations involved in bioluminescent reactions, specifically to study the rate of decay of the light produced when aqueous solutions of enzyme and substrate are added together. This paper deals with a photographic method which has been developed for this study, and with the results which it indicates.

[1] Dubois, R., La vie et la lumière, Paris, 1914.
[2] Harvey E. N., *J. Gen. Physiol.*, 1919, i, 269.

Material.

The material used throughout the study has been the Japanese ostracod, *Cypridina hilgendorfii* Müller. These animals produce one of the brightest, if not the brightest, animal light reactions, which has yet been described. According to Nichols,[3] the maximum brightness of the luminescence in this form is 14.5 to 16 millilamberts, a value higher than that for any fluorescent preparation except some of the uranyl double sulfates. Coblentz has found that a tungsten filament at 2,000°C., that is at about the temperature of ordinary incandescent lamps of the vacuum type, has a brightness of 630,000 millilamberts. *Cypridina* light, therefore, while well up in the scale of brightness for luminescences, is still comparatively faint.

The Cypridinæ have been placed at my disposal in generous quantities by Dr. E. N. Harvey. They have been dried, powdered, and ether extracted, and have furnished a convenient and highly satisfactory material. Aqueous solutions of the enzyme, luciferase, are prepared by extracting *Cypridina* powder with cold water until the luciferin has been completely oxidized, and light has ceased to appear. I have made it a constant practice to extract 5 gm. of the powder with 50 cc. of distilled water. This does not secure a standardization of enzyme strength, for the enzyme, as well as the luciferin, deteriorates with time, although not as rapidly as the latter. It does permit of a measure of control of reaction velocities, producing enzyme strengths of the same order of magnitude. Samples thus extracted are filtered, and the filtrate is used within the following 3 or 4 days. Since chloroform and other preservatives hasten the deterioration of such extracts, I have usually used freshly prepared luciferase to which no chloroform has been added.

Luciferin solutions are prepared fresh for each experiment by the extraction of a sample of the dry powder with boiling water. I usually extract about 2 gm. with 40 cc. of water. After the addition of the water, boiling is continued for a few seconds to destroy the enzyme completely; the solution is then rapidly cooled under the tap. The resulting yellowish solution is allowed to stand for about

[3] Nichols, E. L., *Science*, 1922, lv, 157.

5 minutes until the extracted particles have settled out well. The clear supernatant solution is then carefully decanted and used as needed. I have not attempted to filter such solutions, since I early found that the time used, and exposure to air required, brought about a great diminution in luciferin concentration, due to the spontaneous oxidation already mentioned. This spontaneous reaction has entered in at various points to complicate the problem. It will later be considered in more detail.

The Photographic Method.

Many students of the physics of light have interested themselves in the kinetics of the true inorganic phosphorescences. For a discussion of this field and references to its extensive literature the reader is referred to the memoir of Nichols and Merritt.[4] Direct photometric methods have been universally employed in such work. No previous studies of a similar sort on the time relations of either chemiluminescent or bioluminescent reactions have been reported. In the present work I have attempted to photograph the reaction, by developing a method of photographic photometry.

At first glance it might appear that in such a problem a direct photometric study is feasible and advisable. I was early convinced by a number of considerations that this was not the case in an animal light reaction. The rapidity of the decay in such aqueous solutions as seemed best for study, together with the low intensities of light involved, would make it very difficult, if not impossible, to follow the reaction in this way. The final, and absolutely determining consideration, was that for such studies as those involving the effect of concentration of enzyme and substrate, which I wished to attempt, it would be necessary to investigate two solutions simultaneously. This simultaneous study is necessitated by the fact of rapid deterioration in luciferin solutions, already referred to, so that successive studies of two originally identical portions of such a solution could not possibly give comparable results. A photographic method presented the only possibility for such simultaneous studies.

[4] Nichols, E. L. and Merritt, E., Studies in luminescence, *Carnegie Inst. Washington, Pub. No. 152*, 1912.

Photographic methods for the study of various bioluminescences have been developed by several investigators. Friedberger and Doepner[5] employed a photographic method of determining the intensity of the light coming from a culture of luminous bacteria. They impressed upon their plates a calibration exposure made with a standard lamp of known intensity, and evaluated the densities with the Martens polarization photometer. It would appear that they did not attempt to control the quantity of their calibration light source so as to match that of the organic luminescence in question. Probably the most careful and important use of photographic photometry in such a study is the work of Ives and Coblentz[6] who worked out the spectral energy curve for the firefly, evaluating the intensities along the spectrum by comparing the photographic densities produced by the light of the animal with the photographic effects of a carbon glow lamp, whose spectral energy distribution had been accurately determined.

In the present investigation I have used throughout Eastman cine-negative motion picture film. The spectral sensitivity of this film extends to about $\lambda = 0.59\mu$. This film was obtained from the company before perforation so that the whole width of the film is available for use. The variation in density from point to point in a film, produced by identical exposures, is somewhat less than in plates, in which emulsions are liable to greater variations in thickness. The Eastman laboratories have informed me that the variations to be expected in the developed densities of this film are an average of 3 per cent, and an extreme of 6 per cent. Intensity values calculated from such density readings will vary by somewhat higher percentages. Such deviations are of course unfortunate, but represent a difficulty inherent in all methods of photographic photometry. It must be recognized that great precision is not possible with any photographic method. In the present work I have been able to assure myself that I was working well within the expected error of the film; all records have indicated that this was the case. Every

[5] Friedberger, E., and Doepner, H., *Centr. Bakt., 1te Abt.*, Orig., 1907, xliii,1.

[6] Ives, H. E., and Coblentz, W W., Luminous efficiency of the firefly, *Bull. Bureau of Standards*, 1909, vi, 321. Also see Coblentz, W. W., A physical study of the firefly, *Carnegie Inst. Washington, Pub. No. 164*, 1912.

effort has been made to control the whole manipulation of this film so as to secure the greatest possible uniformity. To facilitate this end only short strips of film have been used, in which great variations in the emulsion would not be expected. The standard length of record finally adopted is 205 mm. long. The whole length of film used, including the records of the reaction, and the calibration exposures, is 250 mm.

After several unsuccessful attempts to construct some optical system which would function to focus the light given out by the reaction upon a moving film, I turned to the use of a simple test-tube container to the side of which is cemented a phosphor-bronze strip in which a vertical slit window has been cut. The two containers used throughout the work were carefully chosen to be of exactly the same bore. The two slit windows were cut in the phosphor-bronze strips by a milling machine, to a width of 1.5 mm. and a height of 6 mm. In width they differ by an error of considerably less than 1 per cent. The two bronze plates are cemented to the containers in identical positions, and were so applied that absolutely clear glass only is apposed to the slit openings. The inner edges of each slit come flush with the glass of the container. All outer surfaces of both tube and brass plate are painted a flat black to prevent transmission or reflection of light. Light can pass from the containers only through the slit windows themselves. The inner edges only of these openings are unpainted, since I wished to avoid the introduction of large errors in slit widths which might arise in the application of such coverings.

With this simple arrangement it is possible to follow the decay of light intensity in reacting *Cypridina* solutions with considerable accuracy. The two containers, mounted upon horizontal supporting bars, adjustable in length, are clamped rigidly upon uprights, and are brought close to the drum of a Zimmermann kymograph. They are adjusted for each experiment so that the line connecting container center and drum center passes through the middle of the slit window, giving a symmetrical and identical orientation for both containers with respect to the drum. The two containers are placed side by side, one of them being fixed about 10 mm. higher than the other. The slit windows are thus presented to two bands of the drum surface

as it revolves, these bands being separated by a narrower strip some 4 mm. wide. An inspection of Plate 1 will make clear the nature of the records resulting from these arrangements.

Records are taken in a dark room. A length of film is wound tightly about the drum, firmly fastened, and then revolved past the slit windows, where it is affected by light issuing from the reacting solutions placed within the two containers. Records are thus taken simultaneously. The Zimmermann kymograph, whose motion is controlled by a governor, gives quite accurate and constant rotation until the spring has nearly unwound. All records have been made with the spring fully wound up at the beginning of the experiment. No attempt has been made to impress any sort of time record upon the film, in view of the high degree of constancy in the speed of rotation. Since absolute measurements of time are of no importance, I have made it a practice to read time in millimeters along the film, and all records presented in this paper are graphed with such values as abscissæ. The actual time of rotation of the drum has been 2 minutes, and the actual speed of the film 4.123 mm. per second.

The containers are brought to a standard distance of 1 mm. from the surface of the drum. This leaves a narrow space through which, in darkness, the film strip can be wound into position about the drum. Under these circumstances there is little diffusion to the sides from the slit windows, but a narrow region to right and left is affected by such diffuse light, and this is undoubtedly a factor affecting the records. Because of it, and of the slit width itself, the effect of the light upon any particular halide grain in the revolving film is not instantaneous. Allowing for such diffusion the exposure time for any particular grain is about half a second. No attempt was made to correct mathematically for these factors of slit width and diffusion, since their effect upon the shape of the curve is slight in any case, and indeed tends to smooth the curve rather than to distort it.

With the containers correctly adjusted, and the film applied to the drum, aqueous luciferin solutions are added to both vessels. I have made it a uniform practice to use 20 cc. of luciferin solution in each container, measured out with a pipette. To each solution, at the proper instant, while the drum is in rotation, there is added 1 cc. of luciferase. The two enzyme solutions have previously been

taken up in a double pipette controlled by a single bulb. Pressure upon this bulb throws an enzyme solution into each tube at exactly the same instant, and with considerable and identical force. In some few experiments I have relied upon the agitation thus produced to secure mixing, and the resulting records have given every evidence of uniformity and consistency. In order, however, after the usual chemical procedure, to insure thorough and continuous mixing of the reactants, I have in most cases stirred both tubes by means of two small motor driven propellers, run at identical speeds, and dipping just below the surface of the reacting solutions. The blades are arranged to throw the surface film continually into the depths. The oxygen supply is thus also replenished, but, as will be seen below, this factor does not appear to affect the reaction velocity.

Eastman cine-negative film is 35 mm. wide. The lower record 6 mm. wide, runs parallel to the lower edge of the film, and at a distance of some 4 mm. from it, the upper record, of the same width, runs through the middle of the film, its lower edge at 4 mm. distance from the lower record. The upper third of the film is thus left blank, and upon it later there is impressed a series of calibration exposures, shortly to be considered.

It can be easily seen that in a photographic study in which quantitative measurements are desired, an accurate knowledge of the relation between the intensity of the incident light and the resulting photographic density is essential. I have made no attempt to secure information of this sort in any absolute terms, although such data would undoubtedly be most interesting if it could be readily obtained. Certain difficulties have made impossible the gaining of such absolute determinations. The whole study has been one of purely relative values.

A host of studies upon the physics and chemistry of the photographic process have given rather extensive information as to the intensity-density relationships. Those who are interested in a detailed discussion of this subject may consult the book of Sheppard and Mees.[7] The curve of blackening of a film or plate assumes a characteristic elongated S-shaped form when the logarithms of the

[7] Sheppard, S. E., and Mees, C. E. K., Investigations on the theory of the photographic process, London, New York, Bombay, and Calcutta, 1907.

intensities of the incident light are plotted as abscissæ, and the resulting densities as ordinates. The term density is defined as the logarithm of the opacity, while the opacity of a film or plate is itself the reciprocal of the transmission. In the determination of photographic densities, therefore, it becomes necessary to secure a method for measuring transmission values; that is, to determine what percentage of a given constant light is able to pass through the density in question. Various methods for such studies have been perfected.

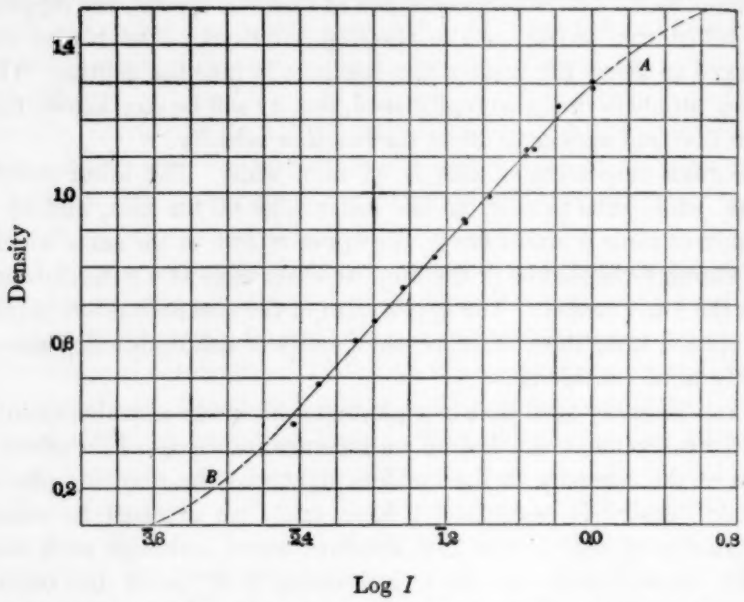


FIG. 1. Curve of blackening. Straight line portion determined by points obtained with the calibration method using *Cypridina* light itself as the light source. Dotted lines indicate diagrammatically how the over-exposure (*A*) and under-exposure (*B*) segments would appear. Abscissæ represent logarithms of light intensities (filter transmissions). Ordinates represent photographic densities.

Perhaps the most popular has been the polarization photometer of Martens.* In my own work I have employed the optical pyrometer, a photometric instrument of great accuracy.

The nature of the curve of blackening can be best understood by reference to Fig. 1, which will also indicate how closely it is possible

to connect light intensity with film density. The dotted portions of the curve represent roughly the two ends of the S, which are not determined by the experimental values obtained in this particular calibration exposure. It will be seen that the curve follows a straight line along a great part of its course, that part, indeed which is determined by the experimental values. This straight line portion of the curve is often spoken of as the normal exposure segment. The upper shoulder of the curve forms the over exposure, the lower heel the under exposure segment. I have endeavored so to control all of the experiments as to hold both moving record and calibration densities to the normal exposure region, although occasionally the densities run out upon the upper shoulder of the S in their higher values.

In photographic literature the contrast of a plate is called gamma (γ). It may be defined as the rate of growth of density with the logarithmic increase of exposure. Graphically it is the slope of the straight line segment of the curve of blackening. The shape of this curve, and especially the slope of the straight line segment, are affected by many factors of which time and temperature of development, kind of developer employed, and the quality of the previously incident light are the most important. In view of the changes thus effected in this curve it is always highly desirable to impress calibration densities upon each individual record, so that such calibrations may progress through all the vicissitudes of development side by side with the regions of the film affected by the unknown intensities of light which are to be evaluated. The two series are then strictly comparable. The paper by Jones[8] (1920) indicates the magnitude of the changes in the value of the gamma produced by developmental variations.

The quality of the incident light affects the slope of the curve of the blackening to some extent, although there appears still to be some dispute among students of the subject as to the magnitude of this effect. Shepherd and Mees (1907) observed differences in the shapes of blackening curves obtained with red and violet light. Some few workers have claimed that gamma does not change at all with different

[8] Jones, L. A., *J. Franklin Inst.*, 1920, clxxxix, 469.

wave lengths (Leimbach,[9] 1909). Abney,[10] Stark,[11] Helmick,[12] Ross[13] and others report more or less marked differences. The recent paper of Ross[13] (1920) is of interest in this connection. His results indicate a very considerable variation, and are of particular interest since they cover in some detail the region of the spectrum in which the *Cypridina* luminescence falls. Thus with identical exposure and development the following values for gamma were obtained: $\lambda =$ 4100Å, $\gamma = 0.74$, $\lambda = 4400$Å, $\gamma = 1.15$, $\lambda = 4800$Å, $\gamma = 1.42$, $\lambda =$ 5100Å, $\gamma = 1.04$. In the light of such data it will be seen that the effect of the quality of the light is indeed considerable.

It is therefore plain that for the evaluation of photographic records produced by unknown light intensities it is of prime importance that the calibration exposures should be produced by light of exactly the same quality. The spectral distribution of light in Cypridina luminescence was studied by Harvey[14] (1920) who reports that visual spectroscopic observation indicates a broad band spectrum extending from $\lambda = 4150$Å to $\lambda = 6100$Å, the broadest distribution yet observed for any bioluminescence. The light of *Cypridina* is therefore decidedly heterochromatic, and its photographic effect is a composite of the effects of all the different wave lengths included in the light. The spectral energy curve has not yet been worked out for *Cypridina*, so it is at present quite impossible to know the relative weights of the different colors involved in producing the composite effect. It is, moreover, impossible to know how to match this photographic effect exactly by using a light filter and a standard lamp, although such a match might conceivably be obtained through a study of the effects of many filters. I early decided that it would be decidedly questionable to employ such a colored filter-standard lamp method of calibration, in the lack of accurate information concerning the energy

[9] Leimbach, G., *Z. wiss. Photo.*, 1909, vii, 157.

[10] Abney, W. W., Treatise on photography, London, New York, Bombay, and Calcutta, 10th edition, 1901, 413.

[11] Stark, J., *Ann. Phys.*, 1911, xxxv, 474.

[12] Helmick, P. S., *Phys. Rev.*, 1921, xvii, 145.

[13] Ross, F. E., Communication No. 95 Research Laboratory, Eastman Kodak Company. Also in *Astrophys. J.*, 1920, lii, 98.

[14] Harvey, E. N., The nature of animal light, Philadelphia and London, 1920, 47.

distribution of the *Cypridina* luminescence. I have sought to achieve the desired results in another way.

The method finally adopted makes use of the *Cypridina* light itself for the impressing of calibration exposures. Two-thirds of the width of the film strip is given over to the moving records. Upon the other third there is impressed a series of fifteen calibration exposures. Plate 1 illustrates several typical records which indicate the results of this method.

The fifteen calibration exposures are impressed simultaneously through a series of fifteen windows in which are placed neutral photographic filters of various known transmission values. Measurement by the pyrometer has indicated that the filters used are very nearly neutral throughout the blue and green regions of the spectrum; that is, throughout that region in which *Cypridina* light affects the film. Absolute neutrality in such screens is impossible of attainment.

The series of filters are mounted in windows cut in a brass strip, wide enough to shield the whole width of the film, and bent in the form of the arc of a circle with a radius of 15 mm. (Fig. 2). At the center of this circle a test-tube 25 cm. in diameter is mounted. The test-tube is painted a flat black with the exception of a segment, *A*, which gives a ring of light at the level of the windows, when reacting *Cypridina* solutions are placed in it. After the moving records of the luminescence have been impressed upon the lower two-thirds of the film, the latter is clamped tightly between the brass strip, *B*, carrying the filters, and a rigid back, *C*, bent to the same curvature. Fifteen spots on the unexposed third of the film are thus brought opposite to the fifteen openings in strip *B*, all spots being equidistant from the ring opening in the test-tube at the center. A concentrated luciferin solution is placed in the test-tube, a motor driven propeller sets the whole solution into rapid rotation, and a concentrated enzyme solution is added. The resulting illumination of the ring opening *A* presents an absolutely identical light source to all of the fifteen windows simultaneously. This illumination is indeed not constant, but falls rapidly through the typical curve of decay characteristic of the reaction. This rapidly diminishing light source, however, exerts an identical effect in every direction at each instant, and the only differential factor in the light which reaches the fifteen spots

of film is the absorption of various known percentages of light by the filters. The method integrates upon the film the effect of a rapidly changing, but always homogeneously radially distributed, light source, over a sufficient length of time to produce photographic densities lying in the same region of the curve of blackening as those caused by the unknown intensities of the moving records which are to be evaluated. I believe that Fig. 1 will give sufficient indication that this is an altogether feasible experimental method. In no case

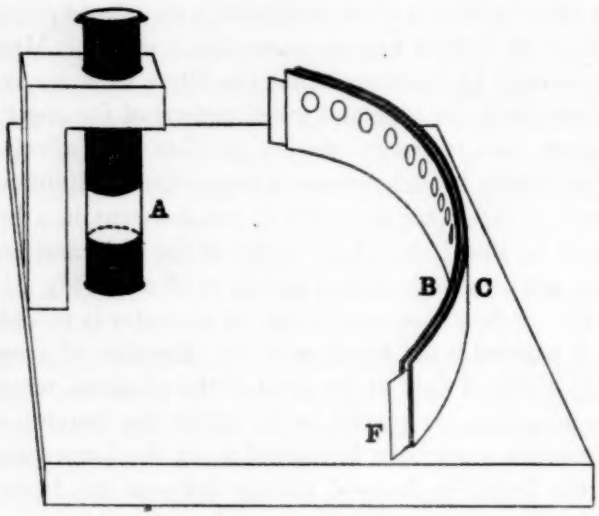


FIG. 2. Diagram of calibration method. The film, *F*, upon the lower two thirds of which the moving records have previously been impressed, is clamped between the rigid back, *C*, and a movable strip, *B*, carrying the fifteen filters of known transmissions. The unexposed third of the film comes opposite to the fifteen openings. Calibration exposures are impressed by light from a luminescent solution ring, *A*, at the level of the filters, giving light with homogeneous radial distribution.

are the density values in error from the mean curve by more than the known error inherent in the photographic material itself. Table I tabulates the known transmission values of the fifteen filters used. Filters 1 to 12 follow a decreasing order of transmission. Filters 13 to 15 give control exposures to check the radial distribution of light.

Windows 1 and 15 carry no filter screens at all, their transmission values being taken as unity. Transmission values for the other windows are expressed as percentages of these two. In practically every experiment the densities produced at 1 and at 15 check well within the known average error of the film.

The whole calibration apparatus is enclosed in a box lined with black felt. All metal parts are painted a flat black, and every precaution has been taken to secure complete absorption of all light

TABLE I.

Transmissions of Calibration Filters.

Filter.	Transmissions.
	per cent
1	100.00
2	65.92
3	48.30
4	43.75
5	26.91
6	20.04
7	13.71
8	9.02
9	6.34
10	5.07
11	3.23
12	2.24
13	19.63
14	42.17
15	100.00

which is not directly incident upon the film through the fifteen openings. There remains only the possibility of cross reflection between the portions of the film exposed through the filters. Calculation has shown that this could not possibly increase the light incident upon other filters by more than 0.5 per cent, and this factor has been neglected.

The determination of the transmission values of filters 2 to 14 requires a brief consideration. The light which falls upon such a screen as is formed by a developed density in a photographic emulsion is transmitted in two ways. A certain percentage passes through by

direct transmission through the spaces between grains. In addition there is a considerable amount of light which is first incident upon the grains themselves, and is then thrown through the screen at various angles by reflection from them. Such additional light forms the "scatter" or diffusion component. The amount of light actually transmitted and available for affecting the film is the sum of these two components thus directly and indirectly transmitted. In the evaluation of such filter transmissions both compounds are automatically taken care of by measuring the percentage of reduction by the filters of the light from a completely diffusing background. For this purpose I have used an opal glass screen illuminated by a 200 watt Mazda lamp run at constant voltage. The filters to be measured are set in immediate contact with this diffusing screen, so that the diffusing surface is in exactly the same position with reference to the filter as is the film at the time of the calibration exposures. The transmission values are then read off with the pyrometer, with which a blue screen is used. It seemed advisable to check the transmission values so determined by some other method in order to be absolutely sure that these values correctly represented the photographic effect. By a photographic method a parallel set of values were obtained which checked the pyrometer determinations within the error of the photographic material. The pyrometer values have been accepted as being probably the more accurate.

The calibration method just described has proven a rapid and satisfactory way of impressing calibration exposures which upon development give a series of densities whose corresponding previously incident relative intensities are accurately known. From these values a curve of blackening similar to Fig. 1 is drawn for each experiment, and densities read along the moving records are referred to the standard curve for evaluation. Extrapolations for short distances in both directions beyond the experimental points are usually used, permitting a range of reading in good records of as much as 100 per cent to 0.5 per cent in light intensity. The average range is about half of this.

In developing films, the records, emulsion side out, are fastened upon a strip of glass of the same length. The whole film is immersed

at the same instant in the developing bath,[15] and rocked back and forth, and up and down at random throughout the whole period of development, to prevent local changes in developer concentrations. I have not attempted to control temperature rigorously, although the temperature of the bath is always about 18°C. Development times are also varied to meet the varying intensities obtained in different experiments. Development is in darkness, with the exception of occasional momentary viewings under the red light.

The time relations of both calibration and moving records have been so controlled as to make the two sets of exposures comparable. It is known that the reciprocity law of Bunsen and Roscoe does not hold for all values of time. It does hold, however, with close approximation if the time is more than a very small fraction of a second, or less than 100 seconds. Exposures along the moving records are for about half a second, while calibration exposures have in no case exceeded 100 seconds. In all records the great burst of light from the calibration tube is over in a very few seconds. The two series of densities may therefore be compared with a good degree of accuracy.

I have entered into some detail in the above descriptions since they appear rather necessary to a complete understanding of the problem, and particularly since the theory and practice of photography in its quantitative relationships is probably little known among biological readers. For the benefit of these readers I will also discuss briefly the methods employed in determining film densities.

The Optical Pyrometer.

The optical pyrometer is a photometer in which the intensity of light emitted from the filament of a small pyrometer lamp is matched against some other light source whose intensity it is desired to measure. Fig. 3 indicates the details of the optical system. In my work I

[15] The following formula was used, diluted with an equal volume of water:

Solution A.	Water	64 oz.
	Hydroquinone	26 gm.
	Sodium sulfite	190 gm.
Solution B.	Water	42.4 oz.
	Potassium carbonate	152 gm.
	Potassium bromide	2 gm.

have used at *A* a special tungsten wire lamp kindly loaned to me by the Nela Research Laboratories. The lamp has a filament made of a single loop of tungsten wire whose diameter is 0.2 mm. An image of a small vertical portion of this filament is focussed by a lens, *B*, upon the filament of a smaller pyrometer lamp, *C*, enclosed in a housing, and the image of both filaments is then viewed through a telescope at *D*. Both lamps are run from storage cells, the amperage being controlled by variable resistances. The intensity of the larger or background lamp is held at a constant value by continual observation of an ammeter in the circuit. The current through the pyrometer lamp is read from a milammeter in this circuit. The relation between intensity of light in the pyrometer filament and the current producing it is determined by reducing the intensity of the constant background by the interposition of various screens or similar devices of known

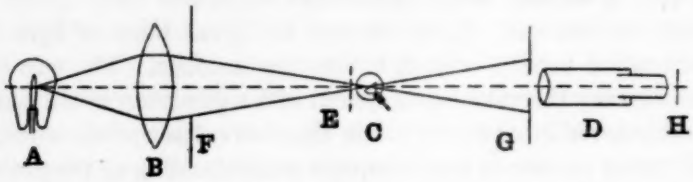


FIG. 3. The optical pyrometer. *A*, background lamp; *B*, objective lens; *C*, pyrometer lamp; *D*, telescope; *E*, film; *F* and *G*, diaphragms; *H*, monochromatic filter.

transmission values. For this purpose I have used the set of sector discs made by the Leeds and Northrup Company. These discs have transmission values of 80, 70, 60, and 50 per cent. At my request the manufacturers made a fifth special disc with a transmission value of 55 per cent. By these discs and combinations of them it is possible to reduce the light to the following percentages of the original value, 80, 70, 60, 55, 50, 35, 30, 25, 20, 15, 10, 5. By reducing the background current two overlapping series of readings connecting the brightness of the pyrometer filament with the current producing that brightness are obtained, extending the range of the calibration to lower transmission values. In each case a disc is rapidly revolved between the background and pyrometer lamp, and the current through the latter is adjusted until the brightness of its filament matches that

of the reduced background. I use a green glass filter at the telescope eye-piece for all density readings, except in the measurement of the calibration filter densities where a similar blue glass screen has been employed.

It is a frequent custom in measuring photographic densities to use a diffusing background similar to that described above for the measurement of the filter transmissions. It is more convenient and equally satisfactory to mount the films to be read in an adjustable holder placed immediately in front of the opening in the pyrometer housing at *E*. Any desired part of record or calibration densities can be interposed between background lamp and pyrometer filament. In this position of the film the light from the background filament is very nearly in focus, the actual focus being about 3 cm. further back at the pyrometer filament itself. The lens and screen at *F* have been so arranged that the image of the filament at the film is 1 mm. wide, and hence a single density determination is made for a strip of film of similar width. At the same time the film is just enough out of focus to prevent the appearance of the developed grains in the telescope, and to give the background at all times a perfectly homogenous brightness.

The transmission values thus obtained represent only the reduction in intensity in the directly transmitted beam. The diffusion component is largely lost. As I wished to compare only one series of photographic densities with another, I have disregarded this diffused light loss. Obviously it makes no difference how such density determinations are made, if the two sets of readings are made in an identical manner. The densities obtained by this method give higher values than would be the case if a diffusing background had been employed. The accuracy of evaluation of the unknown intensities is not affected.

In reading records density determinations have usually been made at every 10 mm. along the film. Such values are calculated from the average of the results of a pair of readings at each point. The permanent photographic record permits rechecking of doubtful determinations. Re-readings usually check to within 1 milliampere with the original readings. Density values can be determined with great accuracy by this method. The most extensive source of error,

therefore, remains the unavoidable errors inherent in any photographic material.

The optical pyrometer is a standard physical instrument and has a considerable literature. Readers interested in fuller discussions of its theory and practice should consult the papers of Forsythe.[16]

EXPLANATION OF PLATE 1.

Reproductions of actual photographic records. The bands are the moving records; the fifteen spots the calibration exposures.

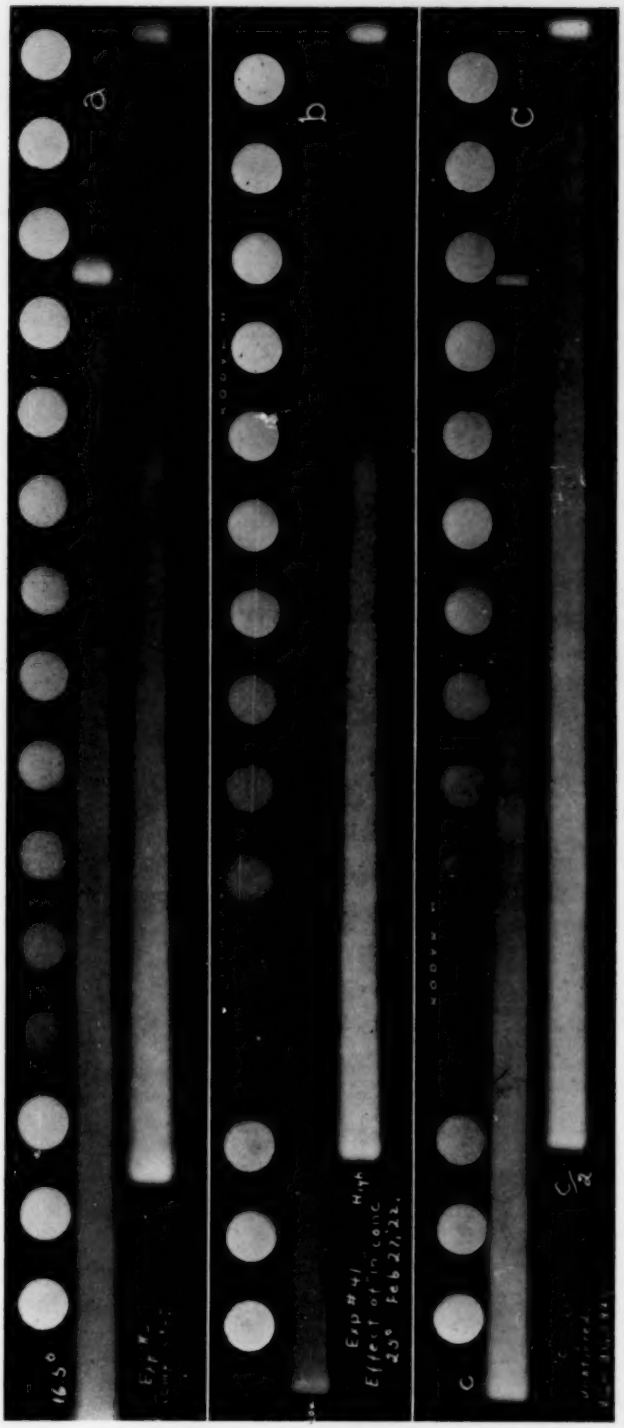
(*a*) Effect of temperature. Temperatures, 16.5°C. and 24°C.

(*b*) Effect of luciferin concentration, the low concentration having been obtained by a brief heating of that solution to 55°C.

(*c*) Effect of luciferase concentrations, C and $\frac{C}{2}$.

---

[16] Hyde, E. P., Cady, F. E., and Forsythe, W. E., *Astrophys. J.*, 1915, xlii, 294; Forsythe, W. E., 1919, xlix, 237; *Am. Inst. Min. and Met. Eng., Bull. No. 153*, 1919, 2547; *J. Op. Soc. Am.*,1920, iv, 305.



(Amberson: Kinetics of the bioluminescent reaction in *Cypridina*. I.)

KINETICS OF THE BIOLUMINESCENT REACTION IN CYPRIDINA. II.

By WILLIAM R. AMBERSON.

(From the Physiological Laboratory, Princeton University, and the Laboratory of Pure Science, Nela Research Laboratories, Cleveland, Ohio.)

(Received for publication, March 22, 1922.)

EXPERIMENTAL RESULTS.

1. Nature of the Curve of Decay in Cypridina Luminescence.

The Initial Flash.—A striking feature, observed in practically every photographic record which I have taken, is the occurrence of a very bright initial flash of light, produced at the instant of the union of luciferase and luciferin solutions, and lasting usually for not more than 2 mm. of film, or half a second. An inspection of the plate will indicate the nature of this flash in terms of its photographic effect. The observed initial light intensities in two good typical records are shown in Tables I and II. These values are to be contrasted with the calculated values in the parallel column, which would be consistent with intensity values read from 10 mm. on to the end of the record, which are connected by a simple mathemetical relationship soon to be discussed. The initial flash is shown graphically in Fig. 1, where it is indicated by the dotted line at the beginning of the curve.

The significance of this flash is problematical. I have been unable to detect any law by which its magnitude may be predicted. I have very often obtained a practical identity in the values for the initial readings on two simultaneous records whose later courses are quite dissimilar. Yet this is by no means a constant observation, and may have no special significance. It should be recalled that the general practice of the physical chemist, in the study of chemical kinetics, is to allow any reaction a brief space of time at least, to straighten out, before measurements are begun.

The Law of Decay.—In an attempt to follow the kinetics of a chemical reaction by the measurement of light intensities emitted

along its curve, it is fundamental to inquire what relation exists between reaction velocity and the resulting light intensity. Trautz[1] from an extensive study of chemiluminescent reactions, but without actual quantitative data, arrived at the conclusion that light intensity is proportional to the velocity of reaction, and therefore a direct measure of it. He based his conclusions upon the observed increase of light intensity in such reacting solutions with the increase of temperature, and upon other similar considerations. Such qualitative observations are by no means adequate to establish such a quantitative hypothesis, but certainly point in the direction of its truth.

In the related field of true inorganic phosphorescence the most comprehensive theory which has yet been proposed is that of Wiedemann and Schmidt,[2] which, in its most general form, supposes that some chemical or physical change is produced in a luminescent material while the exciting radiation is impinging upon it, and that the luminescence which persists when excitation ceases is an expression of the gradual restoration of the original state with the emission of light. More specifically it is generally believed that the effect of the exciting radiation is to split a portion of the luminescent material into equal members of positive and negative particles, probably ionic in nature, and that the luminescence which appears is due to the recombination of these ions at a definite rate. Accurate determinations of the law of decay of light in such materials have given values which fit in well with this hypothesis, if the emitted light is taken to be a direct measure of the number of recombinations at any instant, for it has been found that if the reciprocal of the square root of the light intensity be plotted against time a straight line will connect all the points, and this is the theoretical expectation if two substances, present in equal concentrations, are combining together under the law of mass action. In other words, true inorganic phosphorescences appear to follow the kinetics of a stoichiometric bimolecular reaction.[3] While the theory was originally proposed to explain the form of the

[1] Trautz, M., *Z. physik. Chem.*, 1905, liii, 1.

[2] Wiedemann, E., and Schmidt., C. C., *Wied. Ann.*, 1895, lvi, 177.

[3] It has been found, however, that this is not true at all temperatures, and that some other power of I than $\frac{1}{2}$ may better accord with the experimental observations. See Ives, H. E., and Luckiesh, M., *Astrophys. J.*, 1912, xxxvi, 330.

decay curve, and cannot therefore be considered verified by it, the assumptions are at least the most plausible that can be made at present. The *Cypridina* luminescence is not, however, a phosphorescence but an oxyluminescence.

In the present work I adopted as a working hypothesis this assumption that light intensity is a direct measure of reaction velocity. My

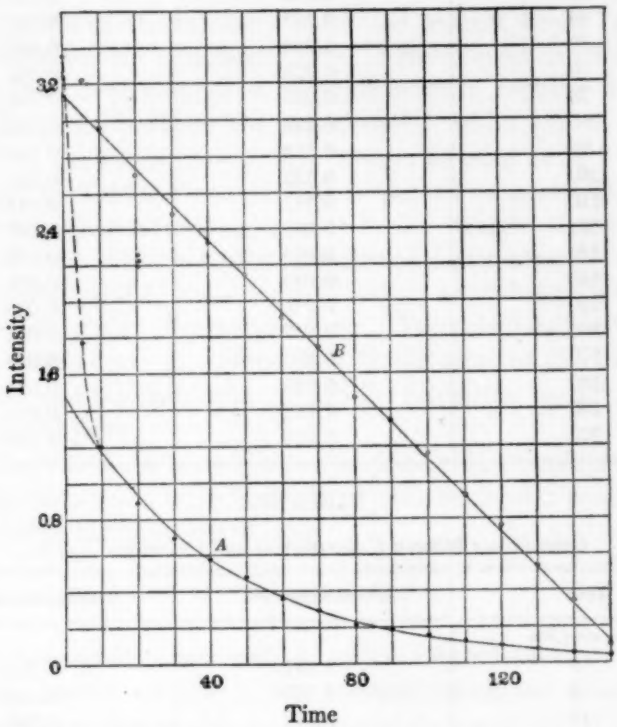


FIG. 1. Decay curve of luminescence in *Cypridina*. *A*. Abscissæ represent time in millimeters along the film; ordinates, intensity of luminescence. *B*. abscissæ represent time; ordinates, logarithm of intensity.

experimental results give a consistent picture when interpreted in terms of such an assumption, and greatly increase the probability of its truth, lending, I believe, quantitative support to the hypothesis of Trautz. Data obtained from a large number of photographic records extending over wide differences in concentration of both

TABLE I.

Coincidence between Experimental and Calculated Values.

Time.	Intensity observed.	Intensity calculated.
mm. along film		
0	3.358	1.479
5	1.778	1.318
10	1.202	1.175
20	0.891	0.929
30	0.692	0.731
40	0.575	0.575
50	0.468	0.460
60	0.355	0.367
70	0.295	0.293
80	0.215	0.232
90	0.184	0.184
100	0.152	0.145
110	0.115	0.115
120	0.096	0.092
130	0.073	0.072
140	0.059	0.058
150	0.044	0.046
160	0.035	0.036
170	0.030	0.029
180	0.022	0.023
190	0.018	0.018
200	0.014	0.014

TABLE II.

Coincidence between Experimental and Calculated Values.

Time.	Intensity observed.	Intensity calculated.
mm. along film		
0	3.311	1.343
5	1.230	1.130
10	0.975	0.948
20	0.650	0.668
30	0.466	0.472
40	0.331	0.331
50	0.246	0.240
60	0.162	0.170
70	0.105	0.120
80	0.083	0.086
90	0.057	0.060
100	0.041	0.043
110	0.031	0.031
120	0.021	0.022
136	0.016	0.015

enzyme and substrate all indicate clearly that in every case after the first bright flash is over, the emitted light dies out by a simple exponential relationship, so that if the logarithm of the intensity of the light be plotted against time a straight line may be drawn through all the points. A graph of the intensity values obtained in a typical decay curve is shown in Fig. 1, these values being the same as those tabulated in Table I. In *A*, intensity is plotted against time; in *B* the logarithm of the intensity is plotted against time. The exponential relationship is unmistakable. I have found this relationship holding in a large number of separate records, totaling 48 to date The coincidence between the experimental values and those calculated from the mean curve is not always as good as in the records tabulated in Tables I and II, but is not to be mistaken. I have plotted all other records submitted in the straight line form only, since this form is best adapted to mathematical analysis.

In Tables I and II the calculated values are those taken from the mean curve of the straight line form. The calculated initial intensities are read from the intersection of the straight line on the zero time axis, and are about one-third of the intensities actually recorded in the initial flash.

The form of the decay curve in *Cypridina* is in complete agreement with the theoretical expectation for a monomolecular reaction. For if, according to the standard form,

$$\frac{dx}{dt} = k(A - X)$$

Where k = velocity constant, A, initial concentration of the single reactant, and X, amount of this reactant which has disappeared in the reaction in time t.

Then by integration

$$k = \frac{1}{t} \log \frac{A}{A - X}$$

$$kt = \log A - \log (A - X)$$

And

$$\log (A - X) = \log A - kt \tag{1}$$

Let I represent light intensity. Then, by the basic assumption

$$I = \frac{dx}{dt} = k(A - X)$$

Taking logarithms,

$$\log I = \log k + \log (A - X)$$

Substitute for $\log (A - X)$ its value from (1)

$$\log I = \log k + \log A - kt$$

or

$$\log I = -kt + \log Ak \tag{2}$$

Equation 2 is the equation of a straight line, in which $\log I$ and t are the two variables, and whose slope is negative and proportional to the velocity constant, whose absolute value may be calculated from it, as we shall see. The experimental values plot in agreement with this form, and are interpreted to indicate that the luminescence proceeds as a monomolecular reaction.[4]

From the work of Harvey[5] and others, we can be sure that oxygen is necessary to the progress of the reaction, in addition to the luciferin itself. It may well be asked, therefore, why the concentration of oxygen is not also a determining factor, and why it does not swing the curve over in the direction of a bimolecular form. The answer appears to be that under the conditions of the experiment, oxygen is always present in excess, and that it therefore does not affect the form of the decay curve.

I have made it a practice to read off the slopes of the straight line plottings directly from the graphical form, and have used these values as if they were the velocity constants, since we are here concerned with relative values, and ratios, and not absolute values. All tabulations of relative values of k have been determined thus directly from the graphs. These values of course depend upon the choice of coordinate scales. The absolute value of k may easily be calcu-

[4] In a note published from the Nela Research Laboratories (Amberson, W. R., *J. Franklin Inst.*, 1922, cxciii, 111.) I stated that the first results of the study indicated a bimolecular form for the decay curve. I have since found that in the first few records my photographic technique was not satisfactory for good quantitative determinations. At the advice of Dr. L. A. Jones I adopted the development technique used by the Eastman Laboratory in their method of photographic photometry, and have since obtained very much better negatives which have consistently indicated the monomolecular form over nearly fifty records.

[5] Harvey, E. N., *Am. J. Physiol.*, 1920, li, 580.

lated if desired. Thus from the decay curve which is tabulated in Table I and graphed in Fig. 1, we derive the absolute value of k as follows:

$$k = \frac{1}{t} \log \frac{A}{A - X}$$

If X becomes $\frac{A}{2}$

Then

$$k = \frac{1}{t} \log 2 \tag{3}$$

The time t needed to reduce the concentration A to half of its value is determined directly from the graph. For log I at time 0, determined from the straight line intersection in Fig. 1, is 0.17.

Then

$$\log \frac{I}{2} = \log I - \log 2$$

$$= \bar{1}.869$$

When I becomes $\frac{I}{2}$, X becomes $\frac{A}{2}$, A reduction of log I from 0.17 to $\bar{1}.869$ corresponds to a time interval of 30.7 mm. of film, or 7.446 seconds of time. Using the usual time unit employed in calculating velocity constants, 1 minute, we find that, as 7.446 seconds is 0.124 minutes, then from equation (3)

$$k = \frac{0.30103}{0.124}$$

$$= 2.43$$

This gives the absolute value of the velocity constant if we are using Briggs' logarithms. The corresponding value of k for the natural system is:

$$k = \frac{2.43}{0.4343}$$

$$= 5.59$$

This absolute value of k will indicate the extraordinarily high speed at which this luminescent reaction is proceeding, even at room temperature.

The Secondary Reaction.—I have mentioned at various places the highly labile character of luciferin solutions which oxidize sponta-

neously in the absence of the enzyme, and without light production. It is not possible at present to state whether this spontaneous oxidation continues side by side with the catalytic oxidation when the enzyme has been added, or how important a part it plays if it be then present. In any case it proceeds at a much lower rate than the catalytic reaction, and does not affect the monomolecular reaction, and hence only affects the value of k in equation (2), without destroying the logarithmic form itself.

It is not possible, of course, to follow the kinetics of this secondary reaction in any direct way since it produces no light. I have attempted to ascertain by an indirect method whether the assumption of a monomolecular form for this reaction is correct. To three tubes containing originally identical quantities of luciferin solution (20 cc.) were added identical luciferase solutions (1 cc.) at successive times, the second addition being 10 minutes after the first, and the third addition 20 minutes later. All solutions were stirred from the instant of addition. The light from these tubes was allowed to fall for 90 seconds from identical distances upon three small neighboring areas of a strip of film. A calibration series was then impressed upon the record and the film developed. In 90 seconds over 99 per cent of the luciferin in such an aqueous solution has been oxidized with light production. It will later be seen that the total light given out when the reaction has gone to completion is probably a linear function of the initial concentration of luciferin. The decrease in total light therefore, in solutions which have stood until the secondary reaction has reduced the initial luciferin concentration, may be taken as a direct measure of such reduction. In one experiment I found that the total light emitted from the second tube, which had stood for 10 minutes, was 52.24 per cent of that emitted by its companion tube run previously. If the spontaneous reaction is monomolecular in character, the third tube run after 20 minutes should have shown a decrease in intensity to 27.29 per cent of the first tube. The actual total intensity observed in this tube was 28.45 per cent which agrees rather well with the expected value. Such data gives fair quantitative support to the hypothesis.

While this secondary or spontaneous reaction has entered in at various points to complicate the problem, it has also proven of value in one part of the study. It is greatly accelerated by a rise in tem-

perature, and I have used this characteristic to prepare luciferin solutions of different concentrations, as will shortly be described.

2. Experimental Errors.

In quantitative work with unstable organic substances, errors are certain to appear, no matter how carefully the manipulation may be

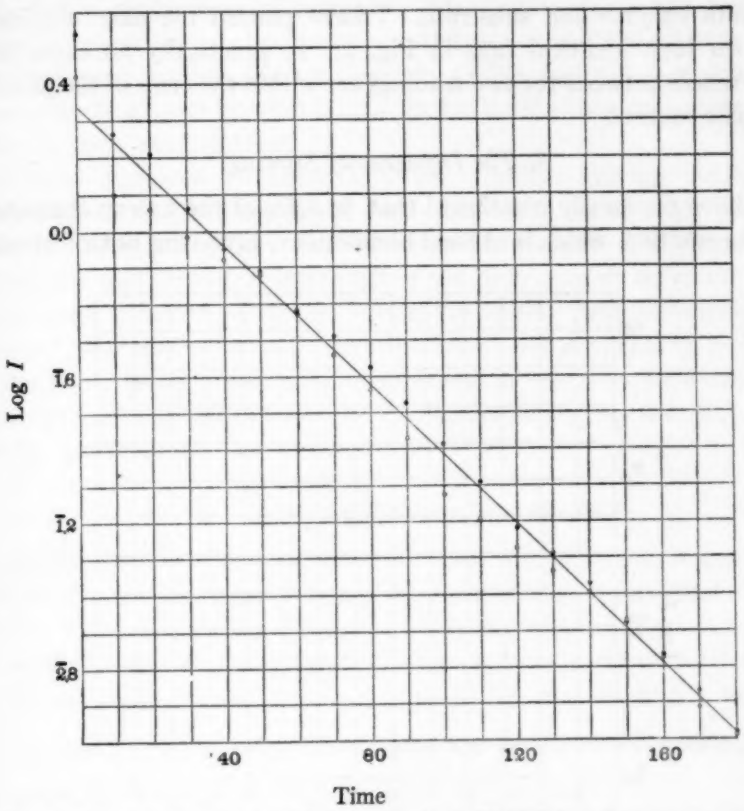


FIG. 2. Identical solutions. The values check within the photographic error. Abscissæ represent time in mm. along the film; ordinates, logarithm of intensity.

controlled. I have made every effort to hold all factors constant, except the one under investigation, but small and uncontrollable variations undoubtedly have been present. It is impossible, for instance to throw all of the enzyme solutions from their containing pipettes. A small amount remains upon the walls. This amount is

usually practically identical in each pipette but variations in enzyme strength of as much as 2 or 3 per cent may be produced in this way.

I have previously discussed the magnitude of the errors known to inhere in the photographic method itself. The question may well be raised, however, as to how closely it is possible to secure identity when simultaneous records are made using identical concentrations of both enzyme and substrate. I have plotted the data obtained in two such identical runs in Fig. 2. In practically all cases the differences between the two readings are within the error of the photographic method.

3. The Influence of Stirring.

I have previously mentioned that, in spite of the known character of the reaction, which is at least bimolecular, involving both luciferin

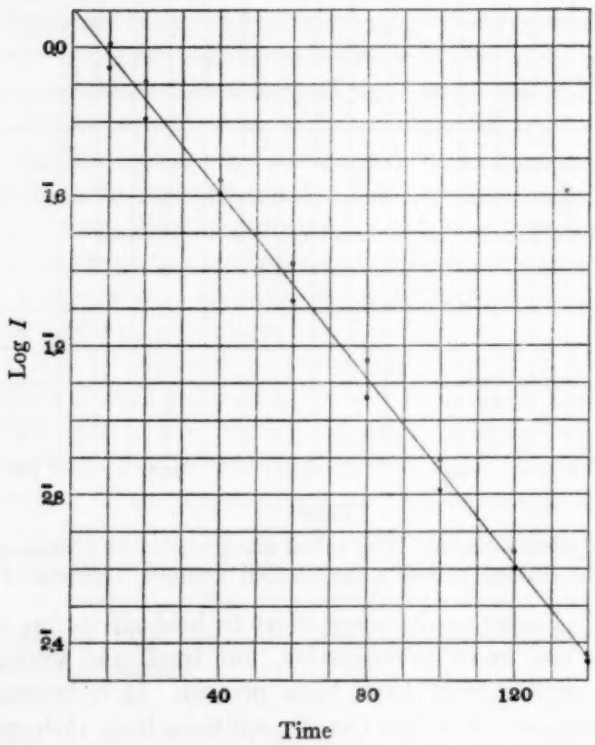


FIG. 3. Effect of stirring. ○ stirred; ● not stirred. Values check within the error of the method. Abscissæ represent time in mm. along the film; ordinates, logarithm of intensity.

and oxygen, the decay curve follows the monomolecular form, because, under the conditions of the experiment, oxygen is always present in excess. The oxygen tension required for a maximum brightness of *Cypridina* luminescence is known to be quite small. Harvey[6] found that the brightness of luminescence in this animal is not affected by decreases in oxygen concentration until the tension has fallen below the value of 53 mm. of mercury. Above this value the oxygen may be considered to be in excess. It has not been possible in the present work to boil out solutions to such a low value of oxygen concentration, or to prevent its rediffusion during the pourings necessary in their manipulation.

I was interested to know, however, whether stirring would in any way affect the form of the decay curve. I found that stirring has no affect, and that two simultaneous records with identical solutions, one stirred, and the other not, follow each other side by side down the curve of decay, within the limits of the photographic error. The results of such an experiment are graphed in Fig. 3. Complete mixing of enzyme and substrate is evidently produced by the force with which the enzyme is added, and stirring does not add to its completeness.

4. The Influence of Enzyme Concentration.

Actual concentrations of materials used are, of course, impossible to ascertain. Accurate relative concentrations of luciferase may, however, be obtained by dilution. Using this method I have studied the effect upon reaction velocity when a certain concentration of enzyme, which we will call C, is reduced by dilution to the values $\frac{C}{2}$, $\frac{C}{4}$, and $\frac{C}{8}$. Both enzyme solutions are added simultaneously to identical luciferin solutions at the same temperature.

Typical records obtained in such a way are graphed in Fig. 4, in which the two enzyme concentration were C and $\frac{C}{2}$. The slope of the straight line plotting gives at once the values of the velocity constants, as already shown in equation 2. The ratio of these slopes

therefore, represents the effect of the dilution upon the reaction velocity. In this case this ratio is 2.17, a slightly higher value than would be expected if reaction velocity were directly proportional to enzyme concentration. Such a variation might well arise from the experimental errors, but in the light of the data tabulated in Table

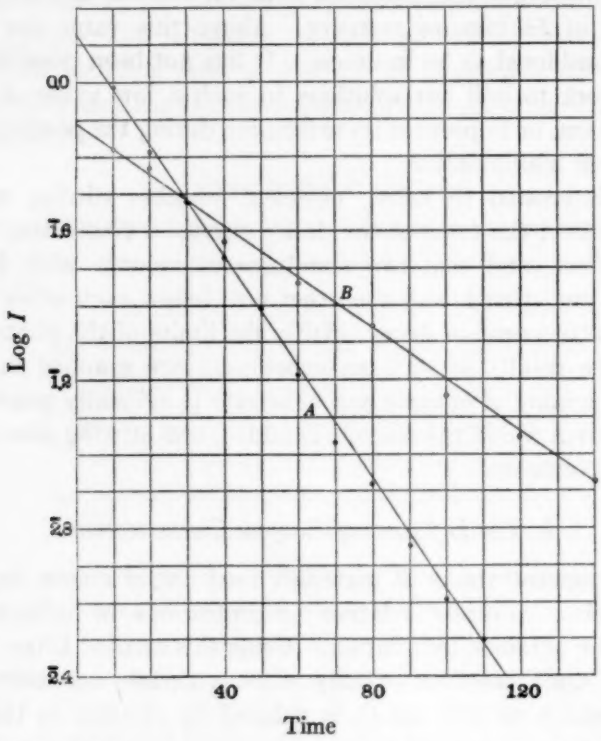


FIG. 4. Effect of enzyme concentration. Abscissæ represent time in mm. along the film; ordinates, logarithm of intensity.

k for A (C) = 1.500

k for B $\left(\frac{C}{2}\right)$ = 0.690

Ratio = 2.17

III, it will be seen that the slope ratios are practically always (one exception) somewhat higher than the expectation for a direct proportionality. It can be stated that the velocity of reaction is very nearly proportional to enzyme concentration.

Upon the assumption of a strict proportionality the theoretical expectation would be that the initial light at zero time would be half as bright for $\frac{C}{2}$ as for C. For from equation 2 if $t = 0$, then

$$\log I = \log Ak.$$

Now if k becomes $\frac{k}{2}$, as a strict proportionality would require, I must become $\frac{I}{2}$, since A is constant. The initial flashes, tabulated in Table III, are seen to be quite erratic, and not at all in line with this

TABLE III.

Effect of Enzyme Concentration upon Reaction Velocity.

Enzyme concentrations.	k	$\frac{k_1}{k_2}$	Initial light.		Initial flash	
			Calculated.	Ratios.	Observed.	Ratios.
C	1.660	2.16	3.020	1.82	Indeterminate.	
$\frac{C}{2}$	0.768		1.660		Indeterminate.	
C	1.168	2.38	1.079	1.92	1.778	1.41
$\frac{C}{2}$	0.490		0.5623		1.259	
C	1.500	2.17	1.340	1.71	3.311	2.04
$\frac{C}{2}$	0.690		0.7762		1.622	
C	1.064	3.94	1.905	3.09	3.162	2.80
$\frac{C}{4}$	0.270		0.6166		1.135	
C	1.007	4.93	1.480	2.32	3.357	2.79
$\frac{C}{4}$	0.204		0.639		1.202	
C	1.195	8.92	0.5598	3.84	0.8185	2.65
$\frac{C}{8}$	0.134		0.1456		0.3090	
C	1.356	9.54	1.393	5.58	2.291	5.56
$\frac{C}{8}$	0.140		0.2495		0.412	

theoretical expectation. Even the values calculated for the initial brightness from the intersections of the straight line plottings upon the zero time axis give poor approximation to the theoretical values. I believe that the presence of the initial flash is responsible in some way for this lack of coincidence, masking, as it does, the fundamental nature of the decay during the first second or two. I consider the very good approximation of the experimental values for reaction velocity with the expectation for a direct proportionality between it and enzyme concentration as being more significant and important than these initial light observations and calculations.

5. *Influence of Luciferin Concentration.*

Returning again to equation (2) we observe that if the value of A, or luciferin concentration, be reduced with all other factors held constant, the value of k is not affected by this reduction, and the slope of the straight line plotting, its graphical counterpart, is also unaffected. In other words, the theoretical expectation is that with two different luciferin concentrations the straight line forms should run parallel to each other.

In my first attempt to investigate this factor I adopted a dilution method similar to that used with success in reducing enzyme concentrations to known lower values. In diluting I first used distilled water, and then later, because this procedure diluted the yellowish pigments always present as well, I diluted with oxyluciferin solutions, boiled until complete oxidation of the original luciferin had been effected. For I found that the yellowish pigments gave a rather high extinction coefficient (about 0.3) for blue light which to the eye was a fair match for *Cypridina* light, and it at once became evident that while dilution with water would presumably not affect the shape of the decay curve, it would affect the magnitude of the light intensities observed all along it.

It appeared therefore that in order to obtain comparable values for two different luciferin concentrations it was necessary to control the pigment concentration. I did this by diluting with oxyluciferin solutions, whose pigment concentration was matched with that of the newly prepared luciferin solution which was to be studied, the two solutions being observed through a colorimeter until the concen-

tration of the oxyluciferin had been reduced by dilution to the proper value. I then prepared C and $\frac{C}{2}$, C and $\frac{C}{3}$, and C and $\frac{C}{4}$ luciferin solutions.

The study of such solutions gave erratic and somewhat curious results. In each separate curve the logarithmic relationship appeared quite definitely, but the expected parallelism in the straight line plottings did not develop. Instead, as Fig. 5 shows, the more dilute

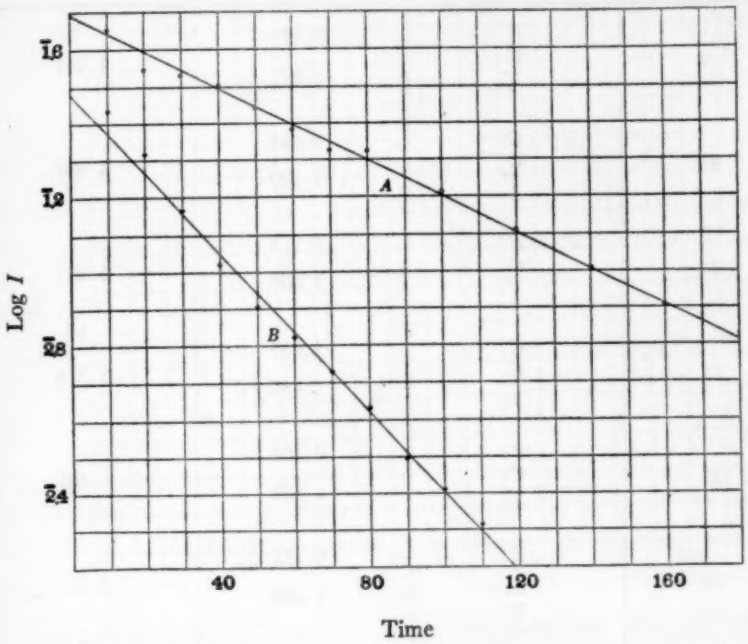


FIG. 5. Effect of dilution of luciferin solutions. Abscissæ represent time in mm. along the film; ordinates, logarithm of intensity.

k for A (C) = 1.080

k for B $\left(\frac{C}{4}\right)$ = 0.484

Ratio = 2.23

solution appeared, in all but one case, to fall through the decay curve with a faster reaction velocity than its companion concentrated solution. Temperature was carefully controlled, and enzyme strengths were of course identical. I have tabulated some of these erratic results

in Table IV. By what must certainly be a chance coincidence the ratio $\frac{k}{k_2}$ comes out very nearly the same for the three C and $\frac{C}{2}$ records studied.

TABLE IV.

Erratic Results Obtained with Dilution of Luciferin Solutions.

Experiment.	Concentration.	k	$\frac{k_1}{k_2}$
4	C	0.570	1.442
	$\frac{C}{2}$	0.822	
33	C	0.845	1.444
	$\frac{C}{2}$	1.220	
34	C	0.778	1.401
	$\frac{C}{2}$	1.090	
35	C	1.060	1.374
	$\frac{C}{3}$	1.456	
32	C	0.704	1.506
	$\frac{C}{4}$	1.060	
36	C	0.484	2.23
	$\frac{C}{4}$	1.080	
37	C	0.910	0.831
	$\frac{C}{4}$	0.756	

I am not able to state what uncontrolled factor has been at work in these dilution experiments to produce this curious acceleration of velocity at lower concentrations of the luciferin. It may possibly be a difference in pH, or in salt concentration. In any case I have been able to determine that these results do not represent the real effect of changing luciferin concentration alone.

In my first attempt to study the possibility of obtaining identical decay curves with identical enzyme and luciferin solutions, I did not use the double pipette which has been described, but started the two reactions successively, the one 16 seconds after the other, so as to place the records side by side upon the film. I found that the resulting decay curves were not superposable, but in the straight line plotting ran parallel to each other. It seemed reasonable to suppose that in the intervening 16 seconds of time the spontaneous

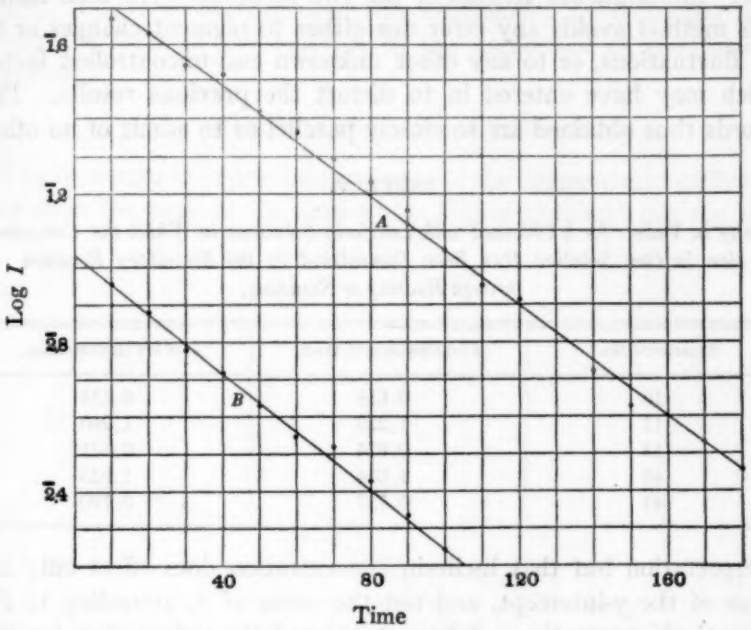


FIG. 6. True effect of different luciferin concentrations. Abscissæ represent time in mm. along the film; ordinates, logarithm of intensity.

k for high concentration (A) = 0.737
k for low concentration (B) = 0.790

oxidation in the second solution might have considerably reduced the luciferin concentration in that solution, with a change of the y-intercept of the straight line, but not of the slope, as above discussed. This experiment furnished the clue to a more satisfactory technique, which does not permit, it is true, of securing accurate previous infor-

mation as to relative luciferin concentrations, but does effect the production of differences in them with all other factors held constant.

The procedure adopted was the rather simple one of increasing the temperature of one sample of a luciferin solution, to hasten the secondary reaction, while its originally identical companion was held at a lower temperature. In the experiment shown in Fig. 6 one solution, giving curve B, was raised to a temperature of 55° for 3 minutes, and then cooled to the same temperature as its companion tube. Simultaneous records of the two solutions were then made. This method avoids any error due either to pigment changes or to pH fluctuations, or to any other unknown and uncontrolled factor which may have entered in to distort the previous results. The records thus obtained are so closely parallel as to admit of no other

TABLE V.

Idenity in Values for k Obtained with Luciferin Solutions in Which the Concentration in One Solution Has Been Diminished by the Secondary Reaction through Heating or Standing.

Experiment No.	k for high concentration.	k for low concentration.
10	0.658	0.635
12	1.220	1.260
13	0.824	0.821
40	1.024	1.023
41	0.737	0.790

interpretation but that luciferin concentration does effect only the value of the y-intercept, and not the value of k, according to the theoretical expectation. I have tabulated the values of k for five such experiments in Table V.

While I have been unable to obtain such records from solutions in which the relative luciferin concentrations are accurately known in advance there is every reason to believe that, since the two plottings are parallel, in accord with expectation, the relative concentrations may also be arrived at from the data. For if A in equation (2) becomes $\frac{A}{2}$, then at zero time, I must become $\frac{I}{2}$, since k is constant; and if A becomes $\frac{A}{4}$, then I becomes $\frac{I}{4}$, and so on. So if, as in Fig. 6,

the logarithmic difference in the values of the two y-intercepts is 0.750, the initial brightness of solution B must have been 17.8 per cent of solution A, and the luciferin concentration must be represented by the same figure.

It follows that the total amount of light emitted from two solutions differing in luciferin concentration will be in direct proportion to those concentrations, for at every instant the light emitted by the one will be the same constant fraction of the light emitted by the other. I have previously used this relationship to determine indirectly the form of the reaction in the secondary or spontaneous reaction.

6. The Temperature Coefficient.

I have made a brief preliminary study of the temperature coefficient, and upon the basis of the data which I have obtained can say that

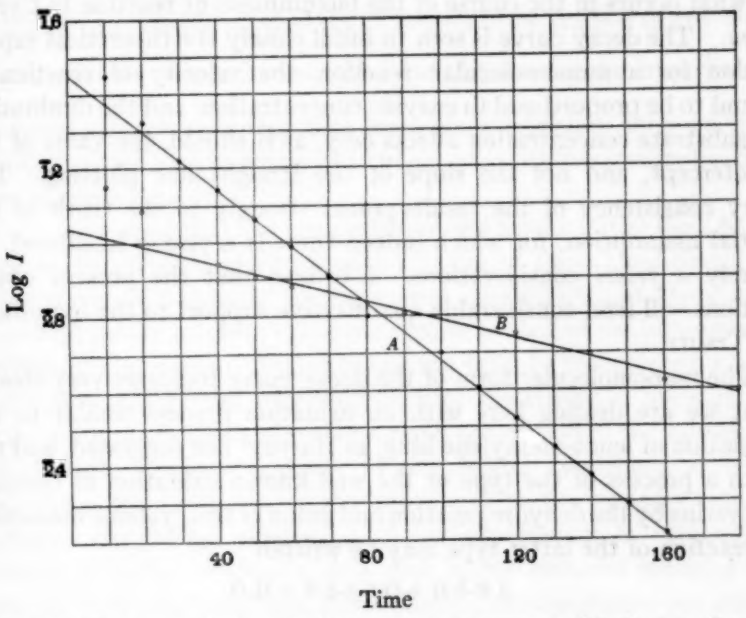


FIG. 7. The temperature coefficient. Abscissæ represent time in mm. along the film; ordinates, logarithm of intensity.

k for A (24°C.) = 0.775
k for B (16.5°C.) = 0.250
Ratio = 3.100

it is quite high for this reaction, the values being about 4.5 for the 15–25° interval, and 3.0 for the 25–35° interval. Fig. 7 will indicate the form of the records assumed in such a determination. From the data of this experiment the value of Q_{10} is calculated by Snyder's[6] formula

$$\left(\frac{k_1}{k_2}\right)^{\frac{10}{t_1-t_0}} = Q_{10}$$

Substitution of the observed values from the experiment in this equation gives $Q_{10} = 4.625$ for the 15–25° interval. The actual value for 28–38° is found to be 2.851.

DISCUSSION.

The following out of the initial assumption of the work, namely that light intensity at any instant is directly proportional to the reaction velocity at that instant, has led to a fairly consistant picture of what occurs in the course of the bioluminescent reaction in *Cypridina*. The decay curve is seen to fulfill closely the theoretical expectation for a monomolecular reaction the velocity of reaction is found to be proportional to enzyme concentration, and the diminution of substrate concentration affects only, as it should, the value of the y-intercept, and not the slope of the straight line plotting. The very consistency of the results points strongly to the truth of the initial assumption, for which indeed there is a strong likelihood, on purely *a priori* considerations. I believe that the present observations will lend considerable quantitative support to the hypothesis of Trautz.

The monomolecular form of the decay curve indicates very clearly that we are dealing here with an oxidation process similar to the oxidation of leuco-methylene blue, as Harvey[7] has suggested, and not with a process of the type of the well known oxidation of cysteine to cystine by the dehydrogenation and union of two cysteine molecules. A reaction of the latter type may be written

$$2\ \text{R-S-H} + \text{O-R-S-S-R} + \text{H}_2\text{O}.$$

Presumably, with oxygen in excess, it would follow a bimolecular form. In the reaction under discussion, however, as in the oxidation

[6] Snyder, C. D., *Science*, 1911, xxxiv, 415.

[7] Harvey, E. N., The nature of animal light, New York, 1920, 128.

of leuco-methylene blue, a single molecule undergoes dehydrogenation according to the form

$$M\text{-}H_2 + O = M + H_2O.$$

The present data are somewhat more difficult of interpretation when we turn to the conditions obtaining during the first few seconds of the reaction, for here we find a bright initial flash out of all accord with the succeeding values, and thereby masking the beginning of the reaction.

As far as I am aware, no report has ever been made, or perhaps has ever been possible, upon the reaction velocity of any organic catalysis during the first few seconds of its course. The reaction under consideration is unique in affording information as to reaction velocity at every instant along its course, and the photographic method which I have employed has given clear evidence concerning the rather striking character of the bright initial flash. The kinetics of no other enzymatic process that I know can be studied in any such direct way. In none other can the $\frac{dx}{dt}$ of the reaction be immediately measured. It appears that this luminescent reaction may be thus peculiarly fitted for the making of further quantitative studies bearing upon enzyme theory.

I am convinced that the occurrence of the bright initial flash has a considerable theoretical significance. It has at times been observed, in the study of inorganic heterogeneous catalysis,[8] that there may occur high initial reaction velocities similar to that of the luminescent reaction in *Cypridina*. Such phenomena may be interpreted as due to the fact that at the very beginning of the reaction, the surfaces of the catalyst are clean, allowing a rapid adsorption of the substrates and a high reaction velocity. These initial conditions speedily vanish, for the collection of the resultants at the surfaces rapidly decreases the active masses, until an equilibrium is established between diffusing resultants, and new active material reaching the surfaces. The reaction then slows down, and straightens out into a consistent form which governs its later course.

[8] Unpublished work from the Department of Chemistry, Princeton University.

It has long been believed by many students of organic catalysis that enzymes and their associated substrates must really be heterogeneous systems, and many facts have pointed indirectly to the truth of this belief. In the present instance there can be no doubt that luciferase, the enzyme concerned, is a colloid, as are most enzymes (Harvey, 1920)[9] and a complex protein at that. There is every reason, therefore, for expecting heterogeneity.

Yet this expectation for enzyme reactions is rarely borne out by the result of kinetical studies. In such heterogeneous systems the rate of diffusion to the surfaces involved becomes an element in the situation, but this factor is usually completely masked by the relative rapidity of this diffusion as compared with the velocity of the reaction itself. Thus Arrhenuis[10] states: "The study of the velocity of reactions in heterogeneous systems indicates that they behave very nearly in the same manner as homogeneous systems . . . . . It depends on the circumstance that the diffusion goes on so rapidly that it does not perturb the chemical processes."

Even in a reaction which proceeds as swiftly as that under consideration, the diffusion rate must be even more rapid and therefore negligible, for the temperature coefficient is high, as usual for enzymatic processes. During the major part of the reaction, therefore, we must be measuring the rate of the oxidation process itself, and no hint of the heterogeneous nature of the system can be derived from the data. The initial flash is, however, in a different category, and I believe that it must be argued that the momentary appearance of this high reaction velocity when the enzyme is introduced is a direct indication of the heterogeneity of the system, to be interpreted in some similar way to that already stated for inorganic heterogeneous catalysis. The details of the mechanism can not yet be stated. It must be admitted that the surfaces of the enzyme particles can not be conceived to be initially completely clean, since, from the manner of their preparation, oxyluciferin is present. The major portion of the surfaces involved may still be free from such reaction products.

Under the experimental conditions of the present work it is certainly not possible to reach the high concentration values which must

[9] Harvey, E. N., The nature of animal light, New York, 1920, 141.
[10] Arrhenius, S., Immunochemistry, New York, 1907, 142.

exist for both enzyme and substrate in the bodies of luminescent forms. We have already seen that even *in vitro*, with comparatively dilute enzyme solutions, the velocity constant reaches a very high value. In those luminescent forms in which the mixing of luciferase and luciferin occurs within the body, concentrations must be much higher, and the reaction must be still further greatly accelerated, so that the light is confined practically to a single momentary flash. Even in *Cypridina* where the luminescent materials are ejected into the sea water, these must be very narrowly localized, and the velocity of reaction and the intensity of the light produced, must be far higher than we can secure in our laboratories. These bioluminescent reactions give a beautiful visual demonstration of the swiftness and efficiency of organic catalysis.

SUMMARY.

1. The decay curve of the light produced in the course of the luminescent reaction in *Cypridina* is, after the first second, in complete agreement with the theoretical expectation for a monomolecular reaction, if light intensity at any instant is assumed to be proportional to reaction velocity at that instant. It is shown that for such a reaction

$$\log I = -kt + \log Ak$$

and that the experimental values satisfy this equation.

2. The first second or two of the reaction is characterized by a brilliant initial flash, whose value is much too high to accord with the succeeding intensities and with the above formula. It is suggested that this initial high reaction velocity is an indication of a heterogeneous system.

3. Identical solutions run simultaneously give decay curves which check within the limits of the photographic error.

4. Stirring does not affect the reaction velocity or the form of the decay curve.

5. Reaction velocity is proportional to enzyme concentration, over the range of concentrations used in the study.

6. Changes in the concentration of the substrate do not affect the value of k, when all other factors are held constant. A diminution

of luciferin concentration results only in a decrease in the value of the y-intercept, Log Ak, the two straight line plottings for two different concentrations being parallel.

7. The temperature coefficient is high, being about 4.5 for the 15–25° interval, and 3.0 for the 25–35° interval.

In conclusion I wish to express my sincere thanks to Dr. E. Newton Harvey, of Princeton University, whose interest in this study has made its completion possible. I also wish to express my deep obligation for valuable assistance upon the physical aspects of the problem to Dr. Edward P. Hyde, Director of the Nela Research Laboratories, and to members of his staff, especially Dr. W. E. Forsythe, Dr. A. G. Worthing, Dr. E. Q. Adams, and Mr. M. Luckiesh, as well as to Dr. Charles Brush, who was kind enough to place a fellowship in the Nela Research Laboratories at my disposal for the summer months of 1921. The experimental methods were developed at that laboratory; the major part of the observations have been made at Princeton University.

A NOTE ON THE ACTION OF CURARE, ATROPINE, AND NICOTINE ON THE INVERTEBRATE HEART.

By A. J. CARLSON.

(From the Marine Biological Laboratory, Leland Stanford Junior University, Stanford University, the Marine Biological Laboratory, University of California, La Jolla, and the Marine Biological Laboratory, Woods Hole.)*

(Received for publication, February 6, 1922.)

The observations recorded in this note were made in 1903–04 as an item in a more comprehensive work on the physiology of the invertebrate heart. These observations were not published, but some points were elaborated in greater detail on the *Limulus* heart, where unusual anatomical relations permit the analysis of the point of action of drugs, and this latter work on *Limulus* was reported in 1906 (Carlson, 1906–07). Having recently, with Dr. Luckhardt, become interested in the action of alkaloids on the nervous tissues in other automatic organs (lungs, gut, and arteries), the author was led to consult the earlier work on the invertebrate heart, and thus came across this item. Feeling that the observations are of some interest to general physiology they are now recorded as written up in 1904, except for the addition of two references (Carlson, 1906–07, 1909) in the bibliography.

The alkaloids used were of Merck manufacture. The species of invertebrates studied were: molluscs (*Octopus, Loligo, Ommastrephes, Mytilus, Mya, Tapes, Platydon, Venus, Pecten, Cryptochiton, Lucapina, Haliotis, Natica, Sycotypus, Aplysia, Bulla, Pleurobranchæa, Montereina, Triopha, Limax, Ariolimax,* and *Helix*), and arthropods (*Palinurus, Cancer,* and *Limulus*).

1. The Action of Curare, Atropine, and Nicotine on Central and Peripheral Ganglia.

The action of alkaloids in the invertebrates has been the subject of numerous studies. The older researches are cited and reviewed in

*I am indebted to the directors of these laboratories for the facilities so liberally extended.

the works of Plateau (1880) and Krukenberg (1880). According to Bert (1867) and Yung (1879, 1881), the action of curare in the crustaceans and in the cephalopod molluscs is the same as in the vertebrates, only less strong. In the lamellibranch molluscs Yung did not obtain a permanent paralysis by curare. Vulpian (1879) states that curare paralyzes both arthropods and molluscs, but its action is not as strong as in the vertebrates. Krukenberg holds that the action of curare in crustaceans and molluscs is mainly on the central nervous system, but there is also paralysis of motor nerve endings. Biedermann (1890) and Fürst (1890) states that curare will paralyze the motor nerve endings in *Lumbricus* but this is denied by Straub (1900).

A drug may cause paralysis by depressing the motor nerve endings or by paralyzing the central nervous system. My own results go to show that the primary action of curare and nicotine in arthropods and molluscs is on the central nervous system and the peripheral ganglia and not on the motor nerve endings in the muscle. The action on the nerve centers is a primary stimulation followed by temporary or permanent paralysis if the dose is of sufficient strength.

There are great differences in the tolerance or degree of resistance to the action of curare in the different invertebrates. The nudibranchs and the pulmonates (excepting *Ariolimax*) are very sensitive to the drug. *Octopus* is far more resistant than the decapods, and of the latter *Ommastrephes* is more resistant that *Loligo*. Of the arthropods, *Limulus* shows the greatest resistance to the drug.

The stimulating action of curare on the nerve centers appears immediately on the application of the solution. In the squid the stimulation results in spasms and tetanus. The primary stimulation is less in evidence in the crustaceans. In the gasteropods it appears in prolonged and extreme contraction of the body muscles. In all the animals studied stimulation of the motor nerves causes contraction of the skeletal and visceral muscles after a dose of curare that completely paralyzed the central nervous system.

Atropine appears to paralyze motor nerve endings to some smooth muscles (*e.g.* lungs, gastrointestinal tract) in vertebrates. The body muscles of arthropods are of the transversely striated type, but the muscle of molluscs approaches more closely to the smooth variety.

My experiments on several classes of molluscs with the view of paralyzing the motor nerve endings in the muscle by atropine have yielded uniformly negative results. So far, then, we know of no drug that will paralyze the motor nerve endings in invertebrates without materially depressing the muscle itself, after previous paralysis of the central ganglia.

2. *The Action of Curare, Atropine, and Nicotine on the Heart Rhythm.*

A review of the literature on the action of the alkaloids on the invertebrate heart discloses considerable disagreement between the results of different observers. Ransom (1884) found that curare in sufficient concentration accelerates the heart rhythm of *Octopus* and *Helix*. According to Yung (1881), curare has sometimes a depressor and sometimes a stimulating action on the heart of lamellibranchs. Plateau (1880) states that curare has a depressor action on the crustacean heart, and according to Dogiel (1877) the drug has no action whatever on the heart of the *Corethra* larva.

Nicotine accelerates the heart rhythm both in crustaceans and in molluscs (Yung, 1881, Plateau, 1880).

Plateau states that atropine has a depressor action on the crustacean heart. Yung and Ransom found that it acts as a stimulant on the molluscan heart. Dogiel states that atropine of sufficient concentration to affect the heart of the *Corethra* larva at all has a depressor action. For an account of the action of these alkaloids on the tunicate heart the reader is referred to the papers by Schultz (1901) and Hunter (1903). My own work does not include the heart of tunicates.

In order to study the action of these drugs on the heart it is, in the first place, necessary to sever the connection of the heart with the central nervous system. All the animals worked on are provided either with inhibitory or accelerator cardiac nerves or both (Carlson, 1909). The action of the alkaloids on the heart when introduced into the intact animal is therefore complicated by their action on the central ganglia or brain and on the peripheral ganglia other than those in the heart. Satisfactory results can for that reason be obtained only on the denervated or excised heart. The solutions of the alkaloids may be applied to the surface of the excised heart and empty heart,

or the heart may be filled with the solution through a cannula. Both these methods were used.

It is furthermore necessary that the solvent is neutral or almost neutral to the heart. Distilled water applied to the molluscan and crustacean heart accelerates the rhythm and produces tonus contractions. It will therefore not do to use distilled water as the solvent. Sea water is almost neutral to the heart of all the marine animals worked on. It has a slight stimulating action but this appears very gradually and only after long immersion of the heart in the sea water. The solution of the alkaloids in sea water will in consequence give fairly accurate results. A still better solvent is the blood plasma of the animals themselves, and this was used in nearly all cases. I was not able to obtain an artificial salt solution that proved to be neutral to the heart of the pulmonates. A solution of the drugs in the blood plasma was the only method available in the work on the snail and the slug heart.

The graphic method was used for recording the change in the heart rhythm.

Solutions of Curare, Atropine, and Nicotine of Sufficient Concentration to Affect Appreciably the Heart Have a Primary Stimulating Action.–The acceleration of the rhythm is followed by depression and, if the concentration of the alkaloids is great, by complete cessation of the rhythm, the heart remaining excitable to direct stimulation. Nicotine is in every case the strongest stimulant. There does not seem to be any great difference between the stimulating action of atropine and that of curare.

The hearts of the various invertebrates studied differ greatly in their sensitiveness to the action of the drugs. In the weakest concentrations of curare the stimulating action appears in augmentation of the rate and strength of the beats, and by gradually increasing the concentration this augmentation passes into a condition of incomplete tetanus. This tetanus may be maintained for 5 to 8 minutes, especially in the heart of lamellibranchs and gasteropods and in the gill ventricles of the squid. The relaxation is gradual and may be accompanied by a feeble rhythm. After the rhythm has been abolished by the action of a strong (1 per cent) solution of curare it can usually be restored by bathing the hearts in plasma. This is not

true for the heart of the crab. A solution of curare strong enough to produce incomplete tetanus of the heart in this species abolishes the rhythm permanently.

The latent period of the stimulating action of curare varies with the sensitiveness of the particular animal to the drug as well as with the mode of application of the solution to the heart. In the molluscs the alkaloid acts more quickly when poured into the cavity of the heart than when applied to the surface of the heart. This is probably due to a difference in permeability. When the solution is applied to the surface of the heart it has to penetrate the epicardium in order to act on the nervous and the muscular tissues, while there is no endothelium lining the heart cavity. In the crustaceans the drug acts equally quickly whether applied to the surface of the heart or introduced into the heart cavity. When the curare solution is applied to the nerve cord on the dorsal side of the heart of *Limulus* the action is practically instantaneous. The crustacean heart is more sensitive to curare than the molluscan heart (with the exception of the heart of *Loligo*). Between closely related molluscs there may be a great difference in sensitiveness. Thus the ventricle of *Ariolimax* continues to beat with an accelerated rhythm for 25 to 30 minutes in a 0.5 per cent solution of curare, a concentration producing cardiac tetanus within a minute in the ventricles of *Helix* or *Limax*.

The primary stimulating action of atropine and nicotine on the heart is to all appearances very similar to that of curare. Nicotine has to be used in dilutions of 1:1,000 to 1:10,000 to exhibit the true stimulating action. In greater concentrations it usually stops the arthropod heart at once without any primary stimulation. A strong solution of curare has sometimes the same effect on the feebly pulsating crab heart. This difference is probably due to differences in permeability.

The strong stimulating action of curare in the invertebrate heart (without exception) is in contrast to the relatively slight action of this drug on the heart of vertebrates. Atropine and nicotine, on the other hand, are strong stimulants also to the vertebrate heart. The stimulating action of the curare solution may in part be due to the action of potassium salts which are present as impurities in commercial curare. I have found that a slight concentration of the potassium chloride in

the blood of *Limulus* has a powerful stimulating action on the ganglion cells in the heart. The solution of these drugs in the isotonic solvents changes the osmotic pressure of these liquids and a slight change in the osmotic pressure of the blood has itself an effect on the heart.

The point of the action of these alkaloids in the heart is not yet known. On the myogenic theory of the heart beat their stimulating effects may be due to action on the accelerator nervous mechanism or to a direct action on the heart muscle. On the neurogenic theory the augmentation of the rate of the beats can hardly be accounted for except by direct action on the local ganglia, while the increased amplitude of the contractions may be due to action directly on the muscle. To answer the question whether these and other alkaloids act on the nervous or on the muscular tissue in the heart or on both, several investigators have studied their action on the embryonic heart on the theory that the heart on the embryo begins to beat before any nervous elements are present. Pickering (1893, 1894–95) found that atropine and nicotine accelerate the embryonic (chick) heart, while strong solutions of atropine depress the rhythm without any primary stimulation. Cyrillo (1901) states that atropine depresses the embryonic heart. This investigator finds, moreover, that the action of the principal alkaloids on the embryonic heart is the same as on the heart of adults, from which he concludes that these drugs act primarily on the heart muscle, their action on the nervous tissue in the heart being entirely of a secondary character.

I have come to the very opposite conclusion, or that the primary action of the alkaloids is on the ganglion cells in the heart and not on the muscle. This conclusion is based on the results on the heart of *Limulus* (Carlson, 1906–07). For this line of study the *Limulus* heart is prepared in the following manner:

The nerve cord on the dorsal side of the heart is extirpated in the first three segments, which leaves this part of the heart free from ganglion cells, the rhythm being maintained by the impulses reaching the muscle from the nerve cord of the middle third of the heart along the lateral nerves. It is desirable to isolate further the ganglionated and the ganglion-free ends of the heart by excision of the heart muscle for a distance of 1 cm. in the third segment. This dissection can be

done without the least injury to the lateral nerves. This makes an ideal preparation. It is a simple matter to apply a solution to the first two segments and absolutely prevent it from getting to the hind portion of the heart and, conversely, to bathe the nerve cord on the posterior end of the heart in a solution without the solution reaching to the first two segments. Any change in the rhythm of the first two segments on application of a drug to the nerve cord of the posterior end of this heart preparation can be due only to a change in the activity of the ganglion cells which maintain the rhythm. And again, the change in the rhythm of the first two segments produced by a solution applied to these segments alone is evidently not an action on ganglion cells but must be an action on the nerves and nerve endings in the muscle or on the muscle itself if we assume that local reflexes play no rôle. For accurate determinations of the changes in the rhythm in the two anterior segments in this preparation, the graphic method was always used.

On this preparation an extended series of tests of the principal alkaloids were made, the results yielding the conclusion expressed above. All the alkaloids tested act on the nerve cord. Some of them, especially veratrin, digitalin, and nicotine act also on the muscle, and it is probable that all of them act on the muscle if in very strong solution; but their action on the nerve cord or ganglion cells is much more rapid and intense, and all the alkaloids, moreover, act on the nerve cord in a dilution which has no or at least a very slight and gradual action on the muscle. Thus a solution of 1 per cent curare or atropine in plasma or sea water stimulates the nerve cord powerfully at the very instant of application, while no change in the rhythm follows its application to the muscle. Further work in this line will probably show that difference in the action of the alkaloids on the nervous and on the muscular tissues in the heart is only one of degree, the ganglion cells being more permeable to the drugs and the muscle cells having lower excitability.

The action of these alkaloids (curare, atropine, and nicotine) on the whole heart of crustaceans and the molluscs is in all essentials the same as their action on the nerve cord or ganglion cells in the *Limulus* heart. The conclusion seems obvious that their action on the crustacean and molluscan heart is also primarily on the ganglion cells.

3. *The Effects of Curare, Atropine, and Nicotine on the Cardioregulative Nervous Mechanism.*

Curare, atropine,and nicotine do not paralyze the cardioaccelerator nerves in the gasteropod molluscs or the intrinsic motor nerves in the heart of *Limulus*. The following experiment on *Ariolimax* may be quoted as typical.

Apr. 4, 1904. Heart of *Ariolimax*.

9.35 a.m. Heart exposed; stimulation of visceral nerve effective. Heart bathed in a 0.5 per cent curare solution in plasma; acceleration.

9.45 a.m. Stimulation of visceral nerve effective. Curare continued.

10 a.m. Stimulation of visceral nerve effective. Curare continued.

10.25 a.m. Stimulation of visceral nerve effective. Curare continued.

10.45 a.m. Accelerator action of nerve partly impaired. Curare solution replaced by blood.

11.10 a.m. Stimulation of visceral nerve accelerates heart.

11.30 a.m. Stimulation of visceral nerve accelerates heart. Blood replaced by 0.5 per cent curare solution. Acceleration of rhythm.

11.50 a.m. Stimulation of visceral nerve has only a very slight action on heart. Rhythm very feeble and slow.

12.15 p.m. Stimulation of visceral nerve still accelerates heart. Curare solution replaced by blood.

1.30 p.m. Heart quiescent. Stimulation of visceral nerve (interrupted current) produced a series of beats in ventricle.

The diminution of the influence of the motor nerves on the heart after prolonged action of these drugs is evidently due to diminished excitability of the heart. The final failure of the accelerator nerves appears only after such a prolonged action of the alkaloids on the heart that the rhythm is almost, if not entirely, abolished. In *Ariolimax* it is possible to produce a series of contractions in the ventricle brought to a standstill by curare, atropine, or nicotine on stimulation of the visceral nerve with the tetanizing current.

I am not in a position to say whether these alkaloids paralyze the accelerator nerves that connect the heart of *Limulus* with the central nervous system, but neither curare, atropine, nor nicotine abolishes the action of the nerve fibers that pass from the nerve cord on the dorsal side of the heart to the heart muscle.

Continued Action of Solutions of Curare, Atropine, or Nicotine on the Heart Abolishes the Influence of the Inhibitory Nerves on the Heart

both in the Molluscs and the Arthropods.—The paralysis of the inhibitory nerves may be only temporary, their function being restored by bathing the heart in blood. The paralysis is not produced except by a concentration of the drugs that causes marked primary acceleration of the heart rhythm. I did not find any essential difference in the paralyzing action of the three alkaloids.

In the lamellibranchs (*Tapes*, *Venus*) the curare solution may act for 20 to 30 minutes before the action of the inhibitory nerves on the heart is abolished. In *Helix* and *Limax* the action is much quicker, or in 4 to 8 minutes. The action is quicker the stronger the solutions. In *Loligo* the injection of a few drops of 0.5 per cent curare or atropine, or 0.1 per cent nicotine in the cephalic vena cava paralyzes the cardioinhibitory nerves in 4 to 5 minutes. I have not succeeded in restoring the function of the nerves in *Loligo* after this paralysis. In *Limulus* the concentration of the drugs must be sufficient to cause great augmentation of the rhythm in order to abolish the influence of the inhibitory nerves on the heart, in which case the paralysis is brought about in a few minutes. In weaker solutions it requires much longer time and may in fact not occur at all.

At what point do these alkaloids act to produce the paralysis of the cardioinhibitory nerves? Atropine paralyzes the vagus fibers in the vertebrate heart, and it is generally held that this action is on the inhibitory nerve endings in the muscle. Nicotine, on the other hand, is supposed to paralyze the synapses of the vagus fibers with the inhibitory ganglion cells in the heart. Turning now to the heart of *Limulus*, it is certain that atropine as well as curare and nicotine act on the ganglion in order to paralyze the inhibitory nerves. If the action of the drugs is confined to the heart muscle and the nerves and the nerve endings in the muscle no paralyzing effects are produced. It would therefore seem that the action of these alkaloids, in abolishing the action of the inhibitory nerves on the heart, is on the ganglion cells in the heart or on the endings of the inhibitory nerves in connection with these cells.

While it is certain that curare, atropine, and nicotine in sufficient concentrations paralyze the cardioinhibitory nervous mechanism in molluscs and arthropods, this action requires, on the whole, a greater concentration of the drugs than that sufficient for the paralysis of the cardiac vagi in the vertebrates.

SUMMARY.

1. The alkaloids (curare, atropine, and nicotine) in molluscs and arthropods stimulate and paralyze the central nervous system and peripheral (visceral) ganglia, but do not paralyze the motor nerve endings to skeletal or visceral muscle.

2. They stimulate and paralyze the denervated heart.

3. They paralyze or block the cardioinhibitory nerves, but not the cardioaccelerator nerves.

4. In the *Limulus* heart these drugs act primarily on the heart ganglion, not on the heart muscle or the intrinsic motor nerve fibers.

BIBLIOGRAPHY.

Bert, P., *Mem. Soc. Sc. Bordeaux*, 1867, v, 115.
Biedermann, W., *Arch. ges. Physiol.*, 1890, xlvi, 398.
Carlson, A. J., *Am. J. Physiol.*, 1906–07, xvii, 177.
Carlson, A. J., *Ergebn. Physiol.*, 1909, viii, 371.
Cyrillo, L., *Molleschott's Untersuch. Naturl.*, 1901, xvii, 229.
Dogiel, J., *Arch. Physiol.*, 1877, 400.
Fürst, M., *Arch. ges. Physiol.*, 1890, xlvi, 367.
Hedbom, K., *Skandin. Arch. Physiol.*, 1898, viii, 185.
Hunter, G. W. J., *Am. J. Physiol.*, 1903–04, x, 1.
Krukenberg, C. F. W., *Vergl. Physiol. Stud. Heidelberg*, 1880, i, 77.
Pickering, J. W., *J. Physiol.*, 1893, xiv, 383.
Pickering, J. W., *J. Physiol.*, 1894–95, xvii, 358.
Plateau, F., *Arch. Biol.*, 1880, i, 695.
Ransom, W. B., *J. Physiol.*, 1884, v, 261.
Schultze, L. S., *Jenaische Z. Naturw.*, 1901, xxxv, 227.
Straub, W., *Arch. ges. Physiol.*, 1900, lxxix, 379.
Tillie, cited by Babák and Chodounsky, K., *Centr. Physiol.*, 1904–05, xviii, 282.
Vulpian, *Compt. rend. Acad.*, 1879, lxxxviii, 1293.
Yung, E., *Compt. rend. Acad.*, 1879, lxxxix, 183.
Yung, E., *Mitt. zool. Station Neapel*, 1882, iii, 97.
Yung, E., *Arch. exp. Zool.*, 1881, ix, 429.

THE EFFECT OF TEMPERATURE ON THE PHOTOTROPIC RESPONSE OF NECTURUS.

By WILLIAM H. COLE.

(From the Biological Laboratory of Lake Forest College, Lake Forest, Illinois.)

(Received for publication, February 6, 1922.)

It is a well known fact that *Necturus maculosus* is negatively phototropic. According to Eycleshymer (1906, 1908, and 1914) Reese (1906), and Pearse (1910), the animal always avoids sunlight in its natural environment as well as in the laboratory. They conclude that the skin is the important receptor for the photic stimulus since eyeless animals behave similarly to normal ones. It is also known that *Necturus* is sensitive to temperatures below 5° and above 25°C. (Reese, 1906; Sayle, 1916). I have found that the animals become more active when transferred from a temperature of 20° to one of 2°C., but that after several hours of exposure to the low temperature, they become very sluggish. As the temperature increases they show increased activity until death ensues between 35° and 40°C. Violent spasmic movements are characteristic of such high temperatures previous to death.

When illuminated, therefore, it may be assumed that the reaction time of *Necturus* will vary according to the intensities of the thermal and photic stimuli. The experiments reported here were performed to determine the effect on the reaction time of variations of the temperature in normal and eyeless animals, and to express it in quantitative form.

The animals were kept in a large aquarium with running water at 20°C. They were thus adapted to light of very low intensity and to a medium temperature. The animals were tested singly in a dark room, where the temperature and the light intensity could be easily controlled. They were placed in a rectangular blackened dish (170 cm. long, 11 cm. wide, and 8 cm. deep), and allowed 15 minutes for acclimatization. At the beginning of each trial, the animal was oriented in the center of the dish, with the longitudinal axes of the animal

and the dish parallel. One of the Mazda glowers was fixed above, so that the beam of light (10 cm. in diameter) centered on the head. When the glower was turned on, the animal crawled posteriorly until the head was out of the beam of light. This interval was designated the reaction time, and was measured by a stop-watch to the nearest second. The light was then turned out, and the animal given a rest period of 1 minute before the next trial began. Four intensities of light were used at four different temperatures on six animals. Ten trials were sufficient to obtain accurate averages before fatigue became apparent.

It was soon discovered that the reaction time of *Necturus* does not change much with the variations of light intensity from 1,875 candle meters to 8,000 candle meters. At a constant temperature of 22°C. and illumination of 1,875 candle meters, the average of sixty trials (ten on each animal) was 2.8 seconds while at 8,000 candle meters it was 3.1 seconds. At 2°C. the average for 1,875 candle meters was 8.1 seconds, and for 8,000 candle meters it was 8.3 seconds. The reaction time was not analyzed as to its exposure and latent periods, which were very short. Crawling began almost immediately after the light was turned on, so that the reaction time is really a measure of the time necessary for the animal to crawl out of the lighted area. Since it is improbable that this constitutes an exception to the Bunsen-Roscoe law, it is evident that in all the trials the maximum amount of stimulating substance was produced in a very short time, even at the lowest intensity. This would be true if the velocity of the photochemical reaction is high enough at 1,875 candle meters. The determination of the exposure period in its relation to the photic intensity was reserved for future investigation. In all the temperature tests only one intensity of light was used, 4,200 candle meters.

From Fig. 1 it will be seen that the reaction time varies inversely to the temperature. Between 2° and 32°C. the temperature coefficient gradually diminishes, as it does in other animals (Hecht, 1919), although its numerical value is not especially significant, since the several chemical processes underlying the reaction time of *Necturus* have not been analyzed. At 32°C. the animal probably moves as fast as its anatomy will allow, without showing the spasmic movements characteristic of temperatures above 35°C. The curve expresses in

definite form the effect of temperature on the phototropic response of *Necturus*, and it may be concluded that the decrease in the reaction time is due to an increase in the velocities of the chemical processes

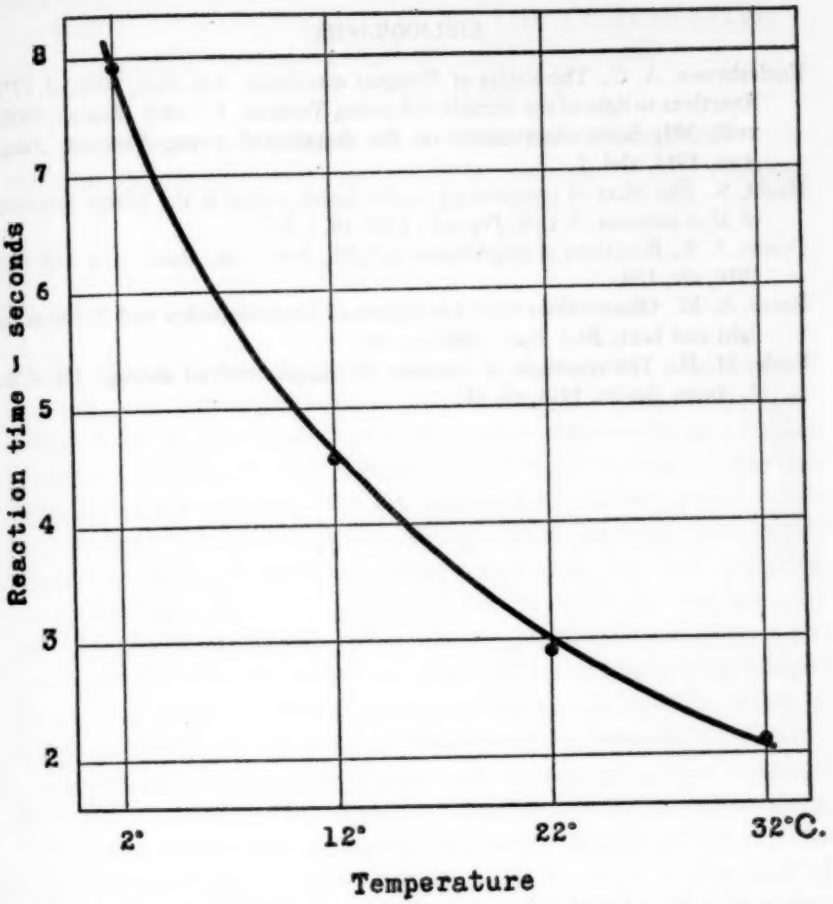


FIG. 1. Relation between temperature and reaction time. Each point is the average of sixty trials; ten on each of six animals. Intensity of light, 4,200 candle meters.

which cause muscular movements, and not to any effect of the temperature upon the photochemical reaction.

48 hours after the eyes had been removed, the six animals were tested again at each of the four temperatures, and with a light inten-

sity of 4,200 candle meters. The averages obtained were almost identical with those from normal animals. It is therefore concluded that the skin is the important receptor for the photic stimulus.

BIBLIOGRAPHY.

Eycleshymer, A. C., The habits of *Necturus maculosus*, *Am. Nat.*, 1906, xl, 123; Reactions to light of the decapitated young *Necturus*, *J. Comp. Neurol.*, 1908, xviii, 303; Some observations on the decapitated young *Necturus*, *Anat. Anz.*, 1914, xlvi, 1.

Hecht, S., The effect of temperature on the latent period in the photic response of *Mya arenaria*, *J. Gen. Physiol.*, 1918–19, i, 667.

Pearse, A. S., Reactions of amphibians to light, *Proc. Am. Acad. Arts and Sc.*, 1910, xlv, 159.

Reese, A. M., Observations on the reactions of *Cryptobranchus* and *Necturus* to light and heat, *Biol. Bull.*, 1906, xi, 93.

Sayle, M. H., The reactions of *Necturus* to stimuli received through the skin, *J. Anim. Behav.*, 1916, vi, 81.

EFFECT OF COCAINE ON THE GROWTH OF LUPINUS ALBUS. A CONTRIBUTION TO THE COMPARATIVE PHARMACOLOGY OF ANIMAL AND PLANT PROTOPLASM.

By DAVID I. MACHT and MARGUERITE B. LIVINGSTON.

(From the Department of Pharmacology and the Laboratory of Plant Physiology, Johns Hopkins University, Baltimore.)

(Received for publication, March 7, 1922.)

INTRODUCTION.

The effects of chemicals on plants have in some respects been studied very thoroughly and in other respects almost not at all. Those drugs or chemicals which have been intensively investigated in this connection are the various salts or more accurately speaking ions which are necessary for the nutrition of various plants. The vast amount of work in plant physiology which has been done on this subject of plant nutrition and plant metabolism has been probably more thorough than the analogous observations on animals. At any rate the plant physiologist is able to produce a more perfect nutritive solution for the growth of plants than the animal physiologist can for the study of animal organs or tissues. While this phase of plant chemistry has received all the attention demanded by its importance, the influence of other chemicals or drugs and poisons on plant life has been barely touched upon. Yet the few contributions along these lines which have appeared, emphasize its importance. If we define pharmacology[1] as some authors do, as ". . . . . the study of the changes induced in living organisms by the administration in a state of minute division of such substances as do not act merely as foods" then the field of what we might term *phyto-pharmacology* is virgin soil. The effects of drugs or poisons on plants have been very little studied. The drugs which have perhaps received slightly more attention in this

[1] Cushny, A. R., A textbook of pharmacology and therapeutics or the action of drugs in health and disease, Philadelphia and New York, 4th edition, 1906.

respect are metallic salts. The effects of these on sprouting and growth have been studied by some authors such as Brenchley (1), Bokorny (2), and a few others. The deleterious effects of some gases have also been touched upon by some authors, such as Crocker and Knight (3), Crocker, Knight, and Rose (4), and others, and the effects of ether on flowers have been investigated by other plant physiologists (5). The influence of that most important class of drugs or poisons known as the alkaloids on the growth of plants, has, however, with the possible exception of nicotine (6) practically been left untouched.

In connection with the comparative study of the effects of cocaine and its decomposition products on various animal organs and tissues carried on by one of the authors, it was thought of interest to inquire into the effects of the same compounds on living organisms belonging to the plant kingdom and an investigation on the subject was accordingly undertaken with the kind cooperation of Professor Livingston of the Laboratory of Plant Physiology of this University. The results obtained were so interesting in themselves, and even more so as compared with the data obtained from animal work, that they are deemed worthy of publication.

Methods.

In the present investigation the effect on the growth of cocaine and its decomposition products were studied, in terms of elongation, of the roots of the seedlings of *Lupinus albus*. This lupine can be very easily germinated and the single straight root of the seedling can be readily measured. The procedure employed in the present experiments was as follows. The dry seeds were soaked over night in tap water at ordinary temperature. On the following day the swollen seeds were planted with the hilum downward in moist, finely ground sphagnum moss. The planted seeds are placed in a thermostat and left at a constant temperature of 20°. On the third day after planting, the roots of the seedlings are of convenient length for measurement and are ready for study. After recording the exact length of a root it is placed in an upright test-tube of hard glass containing nutrient solution, the seed resting on the upper edge of the tube. The solution employed was the so called Shive solution

which contains calcium nitrate, magnesium sulfate, and monopotassium acid phosphate (7). Such a solution was prepared by mixing 10.4 cc. of 0.5 molar solution of calcium nitrate, 30 cc. of 0.5 molar solution of magnesium sulfate, and 36 cc. of 0.5 molar phosphate, with distilled water sufficient to make 1 liter. The normal growth of the lupine rootlets was studied by immersing the seedlings in a mixture of normal Shive solution with an equal part of distilled water. The effect of cocaine and other drugs was studied by dissolving chemicals in distilled water and mixing such drug solutions with equal parts of the normal Shive solution. After measuring accurately the length of each root and placing the seedlings in the control and drug solutions, the whole was again put in the incubator and left at a constant temperature of 20°C., and the effect of various chemicals on the growth of the roots was determined on the following day; that is, at the end of 24 hours. Ten seedlings were placed in the control solution, of one-half Shive solution and one-half water, and ten seedlings were employed for the study of each drug solution. In most of the experiments the volume content of the test-tubes used was about 10 cc. In the case of a few rare and valuable chemicals test-tubes of shorter length, holding about 5 cc., were occasionally employed.

The influence of the following substances, on the growth of lupine roots was investigated: cocaine hydrochloride, sodium benzoate, methyl alcohol, methyl benzoate, ecgonine hydrochloride, benzoyl ecgonine, and various mixtures of these drugs. It is well known that the cocaine molecule can be easily decomposed by hydrolysis, yielding *ecgonine*, *methyl alcohol*, and *benzoic acid*. The anesthetic properties of cocaine in animals are dependent on the chemical union of these three components. A simple mixture of the three will not give the same pharmacological effects as the chemical combination in the form of the cocaine molecule. This is true not only in regard to the anesthetic properties of cocaine but, as previously shown by one of the authors with various collaborators, also holds good in regard to the action of cocaine on the central nervous system (8), on skeletal muscle (9), and on smooth muscle. It was for this reason that it was deemed desirable to inquire into the effect on the lupine root, not only of cocaine itself but also of its various decomposition products.

All of the above substances were studied in various concentrations, the object being to ascertain, in the first place, whether they were toxic to the roots at all and if so to determine their limits of toxicity; that is, the minimal concentrations of the drug which produce a retardation in growth or other toxic effects.

EXPERIMENTAL.

Effect of Cocaine.—Cocaine hydrochloride was employed in the experiments, the chemically pure salt was dissolved in distilled water,

TABLE I.

Relation between Concentration of Cocaine Hydrochloride and Growth of Lupine Roots.

Concentration of cocaine hydrochloride.		Growth increment, for 24 hrs., expressed as percentage of corresponding increment in control test.
mol per liter	*per cent*	
0.2, 0.1, 0.08, 0.06	6.8, 3.4, 2.7, 2.0	No growth.
0.05	1.7	64
0.01	0.34	89
0.005	0.17	100
0.001	0.03	100
0.0005	0.17	100
0.0001	0.003	119, 93
0.00005	0.0017	80, 95
0.00001	0.0003	82, 121
0.000005	0.00017	91, 138
0.0000005	0.000017	90, 116
0.0000001	0.000003	100

and various concentrations of the solution were mixed with equal parts of normal Shive solution, the plants being immersed in the mixture. The results of the experiments are shown in Table I. In the first column are indicated the concentrations of the cocaine hydrochloride expressed as molar, and in the second column are given the equivalents of these concentrations in terms of per cent by weight. In the third column the mean growth increment of the seedlings in the cocaine solutions is indicated as compared with the corresponding normal or control increment, for 24 hours. It will be seen that growth of these roots is affected only by rather strong solutions of cocaine.

The minimal concentration required to inhibit growth completely was 0.06 molar, or about 2 per cent. Solutions slightly more dilute (*e.g.* 0.05 molar, or 1 per cent, allowed a growth increment of 64 per cent), and still weaker solutions did not impair the growth at all. In some of the experiments, indeed, as for instance in concentrations of 0.0000005 molar and 0.0001 molar, there was even a suggestion of a stimulation in growth. This was not, however, definitely established, because of lack of cocaine. The significance of the toxic dose of cocaine for the lupine as compared with that of the same drug for animal tissues will be discussed below.

Toxicity of Ecgonine and Benzoyl Ecgonine.—Cocaine on being hydrolyzed readily yields the base ecgonine. Neither ecgonine nor

TABLE II.

Relation between Concentration of Ecgonine Hydrochloride and Growth of Lupine Roots.

Concentration of ecgonine hydrochloride.		Growth increment, for 24 hrs., expressed as percentage of corresponding increment in control test.
mol per liter	*per cent*	
0.005, 0.0025	0.11, 0.055	No growth.
0.001	0.022	78
0.0001	0.0022	83
0.00005	0.0011	85
0.00001	0.00022	100
0.000005	0.00011	100

benzoyl ecgonine exhibit the characteristic pharmacological properties of cocaine in animals. The effects of these compounds on the growth of lupine are shown in Tables II and III. Table II gives the effects of various concentrations of ecgonine hydrochloride, while Table III exhibits the results of experiments with benzoyl ecgonine. It will be noted that the minimal concentration of ecgonine hydrochloride which produced complete inhibition of growth was 0.0025 molar, or 0.055 per cent. Weaker dilutions ranging from 0.001 to 0.00005 molar, inclusive, permitted growth, though considerably retarding it. In concentrations of 0.00001 molar no deleterious effect on the growth of the roots was evident. It is thus seen that ecgonine is more toxic to lupine roots than is cocaine itself.

An examination of Table III reveals the fact that the toxic dose of benzoyl ecgonine is larger than that of ecgonine itself; in other words the benzoyl ecgonine is less toxic than ecgonine. In order to inhibit growth completely, concentrations of 0.1 molar, or 3.29 per cent, were required. A concentration of 0.1 molar, and lower ones, did not retard

TABLE III.

Relation between Concentration of Benzoyl Ecgonine and Growth of Lupine Roots.

Concentration of benzoyl ecgonine.		Growth increment, for 24 hrs. expressed as percentage of corresponding increment in control test.
mol per liter	*per cent*	
0.1	3.29	No growth.
0.08	2.632	31
0.04	1.316	77
0.01	0.329	100

TABLE IV.

Relation between Concentration of Methyl Alcohol and Growth of Lupine Roots.

Concentration of methyl alcohol.		Growth increment, for 24 hrs., expressed as percentage of corresponding increment in control test.
mol per liter	*per cent*	
1.5	4.8	No growth.
1.0	3.2	27
0.5	1.6	88,73
0.3	0.96	1.05
0.1	0.32	1.19, 1.16, 1.16
0.05	0.16	1.28
0.01	0.03	76
0.005	0.02	75
0.001	0.003	97

growth at all. It is evident that benzoyl ecgonine, unlike ecgonine itself, is somewhat less toxic than cocaine.

Effect of Methyl Alcohol.—The results obtained with methyl alcohol are shown in Table IV. This drug was found to be but little toxic for lupine roots. It required a concentration of 1.5 molar, or 4.8

per cent, to kill the plant, while 1 molar, or 3.2 per cent, gave a growth increment of 27 per cent, or as large as occurred in the controls. Concentrations of 0.1 molar, or 0.32 per cent, gave results suggestive of a stimulation of root elongation.

Effect of Sodium Benzoate and Methyl Benzoate.—The results obtained with these compounds were most interesting, as shown in Tables V and VI. It was found that these esters were both very toxic for lupine roots. Solutions of sodium benzoate as dilute as 0.305 molar, or 0.007 per cent, completely inhibit growth, while methyl benzoate produced complete inhibition in concentrations of 0.0001 molar, or 0.014 per cent.

Effect of Some Mixtures.—Inasmuch as combinations of drugs in animal experiments often give synergistic or antagonistic results, experiments were made with various mixtures of some of the compounds above considered. Four sets of mixtures were prepared and labeled respectively A, B, C, and D. Mixture A consisted of sodium benzoate, methyl alcohol, and ecgonine hydrochloride, the quantity of each used being one-third of the individual lethal dose. Mixture B consisted of sodium benzoate, methyl alcohol, and ecgonine hydrochloride, the quantity of each used being one-third of the individual lethal dose. Mixture C contained one-half of the lethal doses of ecgonine hydrochloride and methyl benzoate, respectively. Mixture D contained one-half of the lethal doses of benzoyl ecgonine and methyl alcohol, respectively. These mixtures were employed in some experiments in their original concentrations, and in other experiments dilutions of these mixtures were tried. It was found, as might have been expected, that Mixture A produced death of the plants. It was furthermore found that, when Mixture A was diluted to one-half the original concentration, it still produced death; in other words, it gave a synergistic effect. Mixture B was also found to be more toxic than was to be expected from a simple summation of its individual components. On the other hand, Mixtures C and D were found to be slightly less toxic than the arithmetical sum of their component effects; in other words, they suggest a slightly antagonistic action of the components against each other.

TABLE V.

Relation between Concentration of Sodium Benzoate and Growth of Lupine Roots.

Concentration of sodium benzoate.		Growth increment, for 24 hrs., expressed as percentage of corresponding increment in control test
mol per liter	*per cent*	
0.174	2.5	No growth.
0.087	1.25	" "
0.07	1.00	" "
0.04	0.625	" "
0.017	0.25	" "
0.0087	0.125	" "
0.003	0.05	" "
0.0028	0.04	" "
0.002	0.03	" "
0.0015	0.022	" "
0.0014	0.02	" "
0.001	0.0144	" "
0.0005	0.007	" "
0.0003	0.004	11
0.0001	0.001	56.2, 62
0.00005	0.0007	67, 66
0.00001	0.0001	11.1
0.000005	0.00007	0.83
0.000001	0.00001	0.97

TABLE VI.

Relation between Concentration of Methyl Benzoate and Growth of Lupine Roots.

Concentration of methyl benzoate.		Growth increment, for 24 hrs., expressed as percentage of corresponding increment in control test.
mol per liter	*per cent*	
0.001, 0.0001	0.136, 0.014	No growth.
0.00005	0.007	13
0.00001	0.0014	30
0.000005	0.0007	74
0.000001	0.00014	79
0.0000005	0.00007	90,90
0.0000001	0.000014	75,93
0.00000005	0.000007	94

DISCUSSION.

The results obtained with cocaine and its various other compounds on the growth of lupine roots are interesting in themselves, but they become much more so when compared with the effects of the same substances on animal organs and tissues. The results of such a comparison are shown in Table VII. In this table the first column indicates the drugs used, the second column expresses the minimal lethal doses of the same for *Lupinus albus*, while the other columns indicate the corresponding effects on animal tissue. In Column 3 are shown the influence of cocaine and its decomposition products, as well as various other mixtures, on the central nervous system. These results are taken from a study by Macht and Bloom (8) concerning the effects of cocaine on the behavior of white rats in the circular maze. In the fourth column the local anesthetic effects of the various drugs are shown. In Column 5 the effects of cocaine and the other drugs on skeletal muscle are described, the data being taken from Kubota and Macht (9). In Column 6 the toxicity of the various compounds for smooth muscle is indicated. These figures are taken from the as yet unpublished studies of the effects of cocaine on the smooth muscle of the bladder and ureters carried on by Macht and Satani. Finally, the lethal doses of cocaine for cats are given in the last column, the figures being taken from Heffter.[2]

It will be noted that, whereas cocaine is by far the most toxic of the substances studied for animals, it is not nearly so toxic for plants. The lethal dose of cocaine for cats is given as 0.02 to 0.04 gm. per kilo, whereas to produce complete inhibition in the growth of the plant a 2.04 per cent solution is required. Ecgonine hydrochloride is quite toxic for animal tissues and, indeed, in the case of skeletal muscle it paralyzed the contractions of the same in 45 minutes, an effect slightly more toxic than that of cocaine (55 minutes). The lethal dose of ecgonine hydrochloride for lupine roots was comparatively very much smaller than that of cocaine, a concentration of 0.55 per cent being sufficient to kill. Benzoyl ecgonine was much less toxic for lupine roots than ecgonine itself, a result which ran parallel to the compara-

[2] Poulssen, E., in Heffter, A., Handbuch der experimentellen Pharmakologie, Berlin, 1920. ii, pt. 1, 145 ff.

TABLE VII.

Comparison between Effects of Cocaine and Related Substances on Lupine Roots, Animal Tissues, etc.

Substance. (1)	Minimal lethal dose for lupine roots. (2)		Effect on central nervous system. (3)	Local anesthetic effect. (4)	Time for paralysis of skeletal muscle. (5)	Effect on smooth muscle. (6)	Lethal dose for cat. (7)
	M	*per cent*			*min.*		*gm. per kilo*
Cocaine hydrochloride ..	0.06	2.04	Depression.	Effect was obtained	55	1:20,000 1:5,000	0.02–0.04
Ecgonine hydrochloride .	0.0025	0.055	None.	None.	45	1:20,000	Over 2.0.
Benzoyl ecgonine.....	0.1	2.5	"	"	Very slight toxicity.	1:750	1.7
Sodium benzoate	0.0005	0.007	"	"	No toxicity.	No effect observed.	0
Methyl alcohol.....	1.5	4.8	"	"	" "	1:50	4.7
Methyl benzoate.....	0.0001	0.014		"			
Mixture A.....	(Synergism.)		None.		Toxic.		
Mixture B.....	(Potentiate.)		"				
Mixture C.....	(Antagonism.)		"				
Mixture D.....	(")				Very slight toxicity.		

tive toxicity of the two for animals. Methyl alcohol was found to be not very toxic for either animal tissues or lupine roots. The most remarkable results obtained, however, were those with sodium benzoate. This salt is very slightly toxic indeed for animal tissues, so that it is employed as a food preservative, yet it was found to be the most toxic of all the compounds studied for lupine roots. It required a concentration of only 0.007 per cent to kill the plants. Methyl benzoate was also very toxic, being second only to sodium benzoate in that respect.

The reason for the remarkable difference just pointed out, in the toxicity of cocaine for animal and for plant tissues, is as yet unknown. The effects of the drugs studied are certainly not attributable to a simple change in the hydrogen ion concentration of the culture media, for experiments made by the authors showed that the Shive solution may be made more or less acid or alkaline without appreciably affecting the growth of the lupine roots. The value of the pH of the normal Shive solution here used was 6.4. More acid and less acid solutions were prepared by varying the quantity of potassium phosphate used, and it was found that the hydrogen ion concentrations varied from 4.4 to 7.2. Within these limits the growth of lupine roots was very little affected.

SUMMARY.

1. The effects of cocaine and its decomposition products were studied on the growth of the young roots of *Lupinus albus*.

2. The results obtained were compared with similar experiments on animal tissues.

3. It was found that, while cocaine is the most toxic of these compounds studied for animal tissues, it was of comparatively low toxicity in respect to its effect on the growth of roots. On the other hand, sodium benzoate, being practically non-toxic for animals, was the most toxic of the compounds studied for the plant roots.

BIBLIOGRAPHY.

1. Brenchley, W. E., Inorganic plant poisons and stimulants, Cambridge, 1914, 110.
2. Bokorny, T., *Biochem. Z.*, 1913, l, 1.

3. Crocker, W. M., and Knight, L. I., *Plant World*, 1909, xii, 83.
4. Crocker, W. M., Knight, L. I., and Rose, *Science*, 1910, xxxi, 636.
5. Grafe, B., Ernährungsphysiologisches Practikum höherer Pflanzen, Berlin, 1914, 392.
6. Otto, R., and Kooper, W. D., *Landw. Jahrb.*, 1910, xxxix, 397.
7. Shive, J. W., *Physiol. Research*, 1915, i, 327-397.
8. Macht, D. I., and Bloom, W., *Arch. internat. Pharmacod.*, 1920, xxv, 8.
9. Kubota, S., and Macht, D. I., *J. Pharmacol. and Exp. Therap.*, 1919, xiii, 31.

STUDIES ON THE REGULATION OF OSMOTIC PRESSURE.

II. The Effect of Increasing Concentrations of Albumin on the Conductivity of a Sodium Chloride Solution.

By WALTER W. PALMER, DANA W. ATCHLEY, and ROBERT F. LOEB.

(From the Department of Medicine of the College of Physicians and Surgeons of Columbia University, and the Presbyterian Hospital, New York.)

(Received for publication, March 23, 1922.)

INTRODUCTION.

It has been shown[1] that the addition of gelatin in increasing concentrations to a 0.6 per cent sodium chloride solution affects the conductivity in two ways, depending on the hydrogen ion concentration. At pH 3.3 the conductivity increases with each added increment of gelatin, whereas at pH 5.1 and 7.4 the conductivity decreases as the percentage of gelatin increases. It was suggested that the addition of gelatin to a solution of sodium chloride has two opposite effects: (1) to increase the conductivity by the addition of ionized gelatin, and (2) to decrease the conductivity by the mechanical interference of the undissociated gelatin molecules. The relative value of these two influences seemed to depend on the degree of ionization of the gelatin, which in turn is dependent upon the hydrogen ion concentration. The experimental results indicated that at pH 3.3 the first effect obtained, while at pH 7.4 and 5.1 the second effect was predominant.

This paper presents similar experiments with another protein; *viz.*, egg albumin. A preliminary determination of the conductivities of pure egg albumin solutions varying in concentrations from 0.8 to 8.7 per cent was carried out as in the case of gelatin.

[1] Palmer, W. W., Atchley, D. W., and Loeb, R. F., *J. Gen. Physiol.*, 1920–21, iii, 801.

EXPERIMENTAL.

The crystalline egg albumin used in these experiments was prepared according to the method described by Hopkins,[2] and recrystallized twice. It was dialyzed until practically free from inorganic salts. The percentage of albumin was determined by drying to constant weight. The hydrogen ion concentration was determined by a gas chain and the conductivity by a Kohlrausch bridge at 25°. Duplicate observations were made in every case. The albumin was brought to the various hydrogen ion concentrations by the addition of NaOH or HCl.

The first experiment (Table I) was performed with pure albumin solutions varying from 1.1 to 8.3 per cent. The conductivity of these

TABLE I.

Conductivity of Pure Albumin Solutions.

Albumin.	Conductivity $\times 10^{-4}$ at pH 3.1.	Albumin.	Conductivity $\times 10^{-4}$ at pH 5.3.	Albumin.	Conductivity $\times 10^{-7}$ at pH 7.3.
per cent		*per cent*		*per cent*	
1.6	9.2	1.1	0.9	1.4	2.1
3.2	14.8	2.2	1.5	2.3	2.8
4.8	19.6	3.7	2.3	3.3	4.1
6.4	23.7	4.7	2.8	6.4	7.8
8.0	27.5	7.4	3.8		
		8.3	4.2		

solutions was determined at pH 3.1, 5.3, and 7.3. The results are plotted in Fig. 1, with the concentrations of albumin as abscissæ and the specific conductivities $\times 10^{-4}$ as ordinates. The curves are reduced to the same scale and plotted at equal intervals above each other. The increase of conductivity with each increment of albumin apparently follows a straight line curve in each instance. The quantitative relations at the various hydrogen ion concentrations are similar to those found in the gelatin solutions.

In a second experiment (Table II) gradually increasing amounts of albumin were added to a 0.6 per cent NaCl solution. Observations

[2] Hopkins, F. G., *J. Physiol.*, 1899–1900, xxv, 306.

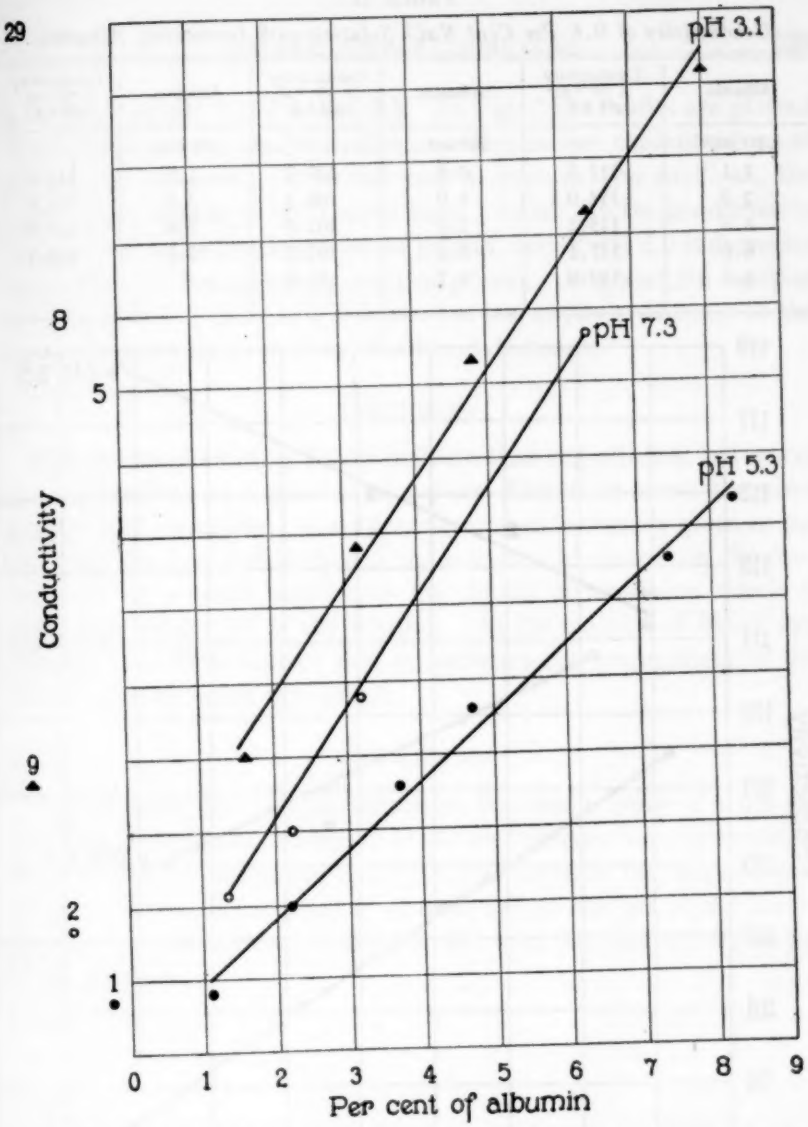


FIG. 1. Conductivities of pure albumin solutions. The abscissæ represent varying concentrations of albumin in per cent, and the ordinates represent specific conductivities $\times 10^{-4}$. The increase in conductivity with increasing concentration of albumin is shown; the increase is greatest at pH 3.1, and least near the isoelectric point (5.3). At pH 3.1, the conductivity increases from 9.2 in 1.6 per cent albumin to 27.5 in 8 per cent albumin. At pH 5.3 the conductivity increases from 0.9 to 4.2 with increase in albumin from 1.1 to 8.3 per cent. At pH 7.3 it increases from 2.1 to 7.8 with an increase in albumin from 1.4 to 6.4 per cent.

TABLE II.

Conductivity of 0.6 Per Cent NaCl Solution with Increasing Albumin.

Albumin.	Conductivity $\times 10^{-4}$ at pH 3.5.	Albumin.	Conductivity $\times 10^{-4}$ at pH 5.0.	Albumin.	Conductivity $\times 10^{-4}$ at pH 7.3.
per cent		*per cent*		*per cent*	
1.1	111.5	0.8	107.9	1.8	110.6
2.9	114.0	1.9	106.4	3.6	108.9
4.8	115.2	3.9	103.8	5.4	107.0
6.6	117.1	5.8	101.2	7.9	105.2
8.3	119.0	8.7	97.0		

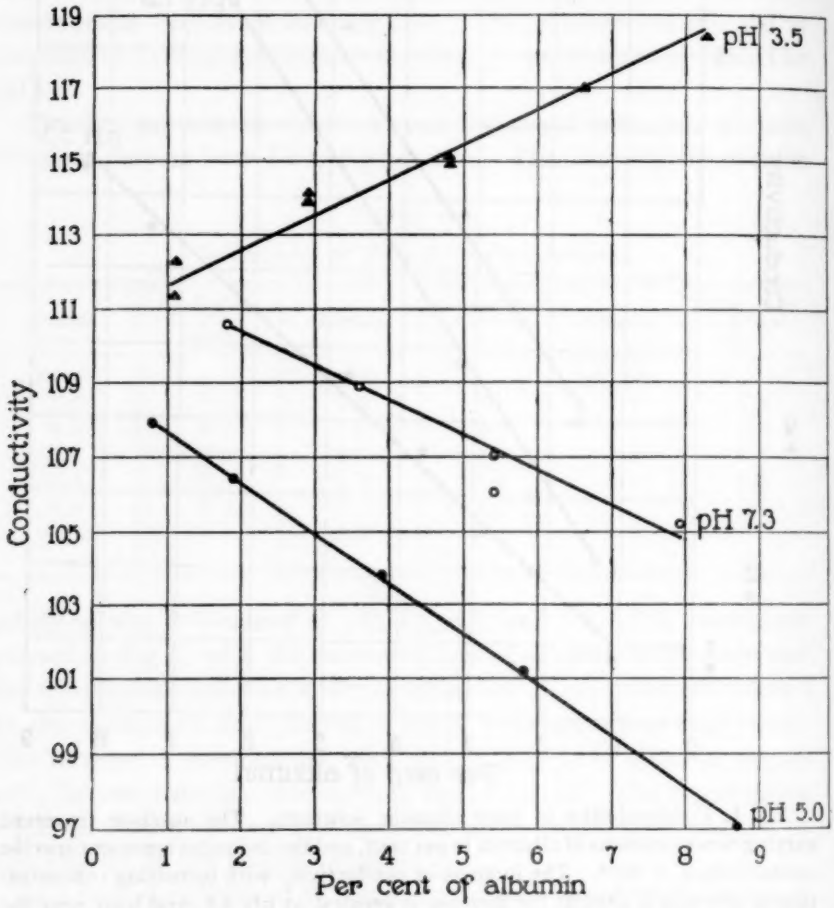


FIG. 2. Changes in specific conductivity ($\times 10^{-4}$) of 0.6 per cent NaCl solutions, with increasing concentration of albumin at various hydrogen ion concentrations. At pH 3.5, conductivity *increases* with increase in albumin per cent. At pH 5.0 and 7.3, conductivity *decreases* with increasing concentration of albumin. This decrease is greater at pH 5.0 than at pH 7.3.

were made at pH 3.5, 5.0, and 7.3. In Fig. 2 the results are plotted on a common scale, conductivities as ordinates and concentrations of albumin as abscissæ. As in the case of pure gelatin solutions, the three curves appear to be straight lines. At pH 3.5 the conductivity increases with the concentration of gelatin, and at pH 5.0 it decreases markedly with increasing amounts of gelatin. At about the reaction of blood, pH 7.3, there is a definite, but less striking, decrease of the conductivity as the percentage of albumin increases.

DISCUSSION.

The results charted in Fig. 2 indicate that egg albumin influences the conductivity of a solution of sodium chloride in about the same manner as does gelatin. Considerable support is thereby given to the idea that the factor determining the influence of protein on the conductivity of a NaCl solution is the degree of ionization which is dependent on the pH of the solution. At the reaction of blood, egg albumin is so little ionized that it decreases the conductivity of the salt solution to which it is added.

CONCLUSION.

Egg albumin, like gelatin, influences the conductivity of a 0.6 per cent NaCl solution in two ways: (*a*) At an hydrogen ion concentration of about pH 3.0, increasing concentrations increase the conductivity. (*b*) Near the isoelectric point of albumin and at the pH of the blood, increasing concentrations of albumin decrease the conductivity of the NaCl solution.

ON THE EQUILIBRIUM CONDITION BETWEEN BLOOD SERUM AND SEROUS CAVITY FLUIDS.

By ROBERT F. LOEB, DANA W. ATCHLEY, and WALTER W. PALMER.

With the Assistance of Ethel M. Benedict.

(From the Department of Medicine of the College of Physicians and Surgeons of Columbia University, and the Presbyterian Hospital, New York.)

(Received for publication, April 6, 1922.)

INTRODUCTION.

There has been much speculation concerning the mechanism by which fluid accumulates in the serous cavities of the body. The presence of ascitic fluid in cirrhosis of the liver has been attributed to compression of the portal circulation. In certain types of renal disease, the retention of fluid has been attributed to some disturbance of kidney function. Fluid accumulations in serous cavities, in inflammatory conditions, have been ascribed to an increased permeability of the capillaries. Thus, it appears, that in the minds of investigators, these various types of edema are classified as unrelated phenomena.

We have made a preliminary study of several physicochemical properties of blood sera and edema fluids simultaneously obtained. The cases studied include heart disease and nephritis with ascites, hydrothorax, and subcutaneous edema; cirrhosis of the liver with ascites; and tuberculous pleurisy with effusion. The results seem to indicate that certain constant qualitative relationships exist between blood serum and fluids in the serous cavities (peritoneal and pleural), regardless of the nature of the fluids or the type of disease.

EXPERIMENTAL.

70 cc. of blood were removed from an arm vein and placed in large centrifuge tubes under oil without exposure to the air. After clotting and centrifugalization, the serum was removed. The patients were tapped immediately after the removal of the blood and the ascitic

TABLE I.

Case.	Diagnosis.	Nature of fluid.	Δ	Specific conductivity × 10^{-4}.	Molecular concentration of Cl × 10^{-4}.	Molecular concentration of HCO_3 × 10^{-4}.	Molecular concentration of Na × 10^{-4}.	Molecular concentration of K × 10^{-4}.	Molecular concentration of Ca × 10^{-4}.	Molecular concentration of glucose × 10^{-4}.	Molecular concentration of urea × 10^{-4}.	Non-protein N per 100 cc.	Protein content.
												mg.	*per cent*
Cl.	Cirrhosis.	Serum.	0.498	113.7	100.0	27.5	124.7	4.2		7.2		26	6.8
		Ascitic fluid.	0.509	132.5	108.2	26.3	138.2	2.4		7.2		24	0.9
K.	"	Serum.	0.490	117.7	103.8	23.8	133.8	4.7	2.4	8.2	7.8	33	5.2
		Ascitic fluid.	0.488	131.9	109.0	23.8	126.6	2.0	2.3	9.0	8.2	31	0.8
Co.	"	Serum.	0.518	115.9	105.6	26.4	112.2	2.7		7.3		30	7.0
		Ascitic fluid.	0.507	135.2	113.7	25.6	138.6	2.3		9.6		27	0.9
D.	Nephritis.	Serum.	0.535	127.5	110.9	27.0	149.4	3.5		6.3		38	5.0
		Chest fluid.	0.535	144.7	119.7	26.6	147.7	1.7		6.9		34	0.6
P.	Cardiorenal.	Serum.	0.549	125.5	115.3	16.9	139.8	4.7		5.1	19.0	74	6.0
		Ascitic fluid.	0.549	135.8	121.1	16.2	140.8	3.2		5.2	19.0	68	3.3
McF.	Cardiac.	Serum.	0.533	120.6	105.6	28.4	140.0	4.5		5.6		23	7.3
		Ascitic fluid.	0.534	130.0	112.7	28.4	141.3	3.1		6.8		25	4.5
H.	Tuberculous pleurisy.	Serum.	0.515	115.9	101.2	29.9	138.7	4.4		5.5		22	7.1
		Chest fluid.	0.512	121.0	104.4	29.3	142.1	2.7		5.9		21	5.6
Limit of error of analytical methods used.....					0.9	0.7	6.1	0.3		0.4	0.3	2.0	

or chest fluid was received under oil, to avoid any marked change in hydrogen ion concentration through loss of CO_2. The following physical and chemical determinations were made on the blood sera and the edema fluids; freezing point depression, specific conductivity, Cl, HCO_3, Na, K, glucose, non-protein nitrogen, protein per cent (by Kjeldahl and refractivity), and, in certain cases, urea and Ca. A description of the methods employed, and other details, will be published later. The results of these observations are collected in Table I.

In four cases, serum was placed in a thin collodion sac with a capacity of about 6 cc. and immersed in a bottle containing 250 cc. of edema fluid. This bottle was kept at 25°C. A manometer was placed in

TABLE II.

Case.	Diagnosis.	Molecular concentration of K × 10^{-3} in fluid.	Molecular concentration of K × 10^{-3} in serum.		Molecular concentration of Cl × 10^{-3} in fluid.	Molecular concentration of Cl × 10^{-3} in serum.		Protein in serum.	
			Before dialysis.	After dialysis.		Before dialysis.	After dialysis.	Before dialysis.	After dialysis.
								per cent	*per cent*
P.	Cardiorenal.	3.2	4.7	5.2				6.0	5.9
Co.	Cirrhosis.	2.3	2.7	2.6	113.7	105.6	107.2	7.0	6.7
H.	Tuberculous pleurisy.	2.7	4.4	3.9					
McF.	Cardiac.	3.1	4.5	4.6	112.7	105.6	109.8		

the collodion sac, and the serum level was so adjusted that there would be little or no change in level with the establishment of equilibrium. After 18 hours, the contents of the sac were analyzed for protein per cent, K, and Cl. The results of these analyses were compared with the original concentrations and with those of the edema fluid as shown in Table II. The bottle containing the edema fluid and collodion sac was kept closed with a rubber stopper and a soda-lime tube to prevent change in hydrogen ion concentration.

DISCUSSION.

From the data in Table I, the following relationships between blood serum and edema fluids are apparently constant. (*a*) The freezing

point depression of serum and of edema fluid is the same—within the limit of error of the method when applied to physiological solutions. (*b*) The conductivity of the edema fluid is always higher than that of the blood, but the greater the protein content of the edema fluid, the closer the conductivity approaches that of the serum. This is in accord with experiments on pure protein solutions, in which it was found that, at the pH of the body fluids, the higher the protein content, the lower the conductivity.[1] (*c*) The chloride content of the edema fluid is always higher than that of the serum. (This is at variance with findings of Epstein.[2]) This difference of Cl concentration in blood and edema fluids diminishes, in general, as the protein content of the edema fluid increases. (*d*) The concentration of potassium is greater in the serum than in the edema fluid. (*e*) The concentrations of HCO_3, Na, sugar, non-protein nitrogen, and Ca and urea, where these were determined, are approximately the same, in the determinations made thus far.

The experiments on the dialysis of serum against edema fluid reported in Table II, demonstrate that no new equilibrium is established when the two fluids are separated by a simple collodion membrane. The relatively high concentration of potassium inside and the relatively high concentration of chlorine outside the membrane are but slightly changed. The results suggest that these interesting relationships depend on a simple membrane equilibrium and are not entirely due to properties peculiar to living protoplasm.

CONCLUSIONS.

1. Comparative studies of blood serum and edema fluid from the same individual indicate that, regardless of the pathological condition present, whether the fluid be "transudate" or "exudate," certain definite qualitative chemical relations obtain.

2. The chief feature of these relations is that the edema fluid contains more Cl and less K than the blood serum; while the Na, HCO_3, Ca, urea, glucose, and non-protein nitrogen exist in approximately

[1] Palmer, W. W., Atchley, D. W., and Loeb, R. F., *J. Gen. Physiol.*, 1920–21, iii, 801; 1921–22, iv, 585.

[2] Epstein, A. A., *J. Exp. Med.*, 1914, xx, 334.

the same concentrations in the serum and in the edema fluid. The freezing point is also the same in both fluids, while the specific conductivity is constantly higher in the edema fluid.

3. The above mentioned variations between the edema fluid and the serum appear to be related to the difference in the concentration of protein in the two solutions.

4. The relationships between blood serum and edema fluid seem to result from a simple membrane equilibrium, influenced in part by the proteins present.

THE COLLOIDAL BEHAVIOR OF EDESTIN.

By DAVID I. HITCHCOCK.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, April 1, 1922.)

I.

INTRODUCTION.

It has been shown in a series of papers by Loeb[1] that the physical, chemical, and so-called colloidal properties of solutions of the proteins, gelatin, egg albumin, and casein, can be simply explained by two general principles. The first of these is that proteins are amphoteric electrolytes, reacting stoichiometrically with acids and bases to form salts capable of electrolytic dissociation; the second is the principle of Donnan's membrane equilibrium,[2] which is set up when two solutions are separated by a membrane impermeable to one ion of one of the solutions.

The present investigation was undertaken with the object of finding out whether these laws would explain the behavior of solutions of a protein of a different class; namely, a globulin. The globulin selected for the purpose was edestin; and it was found that its solutions obeyed the same laws which had been shown to apply in the case of the other proteins.

The edestin used in these experiments was prepared from ground hemp-seed by the method of Osborne,[3,4] with slight modifications. The hemp-seed meal was extracted three times with 10 per cent sodium chloride solution at 60°C., without previously extracting the oil. The edestin was precipitated by dilution and recrystallized once from sodium chloride solution according to Osborne. The substance was

[1] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922; *J. Gen. Physiol.*, 1918–22, i–iv.

[2] Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572.

[3] Osborne, T. B., *J. Am. Chem. Soc.*, 1902, xxiv, 28, 39.

[4] Osborne, T. B., *Abderhalden's Handb. d. biochem. Arbeitsmethoden*, 1910, ii, 289.

freed from sodium chloride by washing repeatedly with 50 per cent alcohol, and was partly dried by washing with absolute alcohol and then with ether, which left it with a moisture content of 10.1 per cent as determined by heating to constant weight at 110°C. The dry powder was found to contain 18.4 per cent of nitrogen, as determined by a micro-Kjeldahl method accurate to about 1 per cent, whereas Osborne[4] reported 18.69 per cent of nitrogen. Under the microscope the preparation appeared to have the form of fragments of crystals.

The isoelectric point of edestin was given by Rona and Michaelis[5] as at a hydrogen ion concentration of 1.3×10^{-7} (pH 6.89). This was the point of maximum precipitation obtained by using a series of phosphate buffers.[6] Attempts were made to determine the isoelectric point of the present preparation by similar methods, but the point of maximum precipitation seemed to vary with the buffer used. Most of the values obtained, however, lay between pH 5 and 6. Measurements of the osmotic pressure developed in collodion bags by suspensions of edestin in various concentrations of very dilute sodium hydroxide indicated a minimum between pH 5 and 6. Electrophoresis experiments with suspensions of this edestin were made by Dr. John H. Northrop of this laboratory, who also obtained different results with different buffers, which indicated, however, that the isoelectric point appeared to lie between pH. 5.5 and 6.0. The pH of suspensions of this edestin in distilled water was found to be 5.0.

II.

Titration of Edestin with Acids and Bases.

Titration curves of solutions of edestin in acids and bases were obtained in the region where 0.45 gm. could be almost completely dissolved in 100 cc.; *i.e.*, below pH 5.0 and above pH 9.0. Solutions were prepared containing different quantities of acid or alkali but of the same concentration with respect to edestin, and the pH values were

[5] Rona, P., and Michaelis, L., *Biochem. Z.*, 1910, xxviii, 193. The edestin used by Rona and Michaelis was apparently a different substance from the present preparation, since they stated that only a small fraction of 1 per cent was soluble in 0.1 N H_3PO_4, while the edestin used in this investigation was completely soluble to the extent of 1 per cent or more in 0.1 N H_3PO_4.

[6] See also Michaelis, L., and Mendelssohn, A., *Biochem. Z.*, 1914, lxv, 1. Using acetate buffers they obtained a value of 2.5×10^{-6} (pH 5.60) for the isoelectric point of a different edestin preparation.

ascertained by means of the hydrogen electrode, using a salt bridge of saturated potassium chloride and a saturated potassium chloride calomel cell. The measurements were made at 33° ± 0.5°, and were referred to 0.1 M HCl as a standard, its pH being taken as 1.036.

The titration curve obtained with 0.45 per cent solutions of edestin in phosphoric acid is given in Fig. 1, Curve I. As a means of determining how much of the acid was combined with the edestin, Curve II was obtained by measuring the pH of mixtures of phosphoric acid and water alone. Curve III, which gives the amount of phosphoric acid apparently combined with the edestin, was obtained by subtracting the ordinates of Curve II from those of Curve I at corresponding pH values. Since phosphoric acid was known to be a weak acid, it was suspected that some of the acid indicated by Curve III to be combined with the edestin might really be present as un-ionized molecules, due to the common ion effect of the ionized edestin phosphate. Accordingly a calculation was made of the first ionization constant of phosphoric acid at 33°, from the data used in plotting Curve II. The results are as follows:

pH	$k = \frac{[H^+][H_2PO_4^-]}{[H_3PO_4]}$
2.72	0.0101
2.47	0.0100
2.29	0.0094
2.18	0.0092
1.93	0.0096

These values may be compared with that found by Abbott and Bray at 18°, 0.011.[7] Their constancy indicates that over this range of pH the acid is really monobasic. Accordingly this ionization constant, roughly 0.01, was used to calculate the amount of edestin phosphate actually present, assuming complete ionization of this salt. This was done as follows:

Let c = total concentration of H_3PO_4 (ionized, un-ionized, and present as edestin phosphate),
x = concentration of H_2PO_4 from edestin phosphate (assumed to be completely ionized),
h = concentration of H^+ = concentration of H_2PO_4 from H_3PO_4,
k = primary ionization constant of H_3PO_4,

[7] Abbott, G. A., and Bray, W. C. *J. Am. Chem. Soc.*, 1909, xxxi, 760.

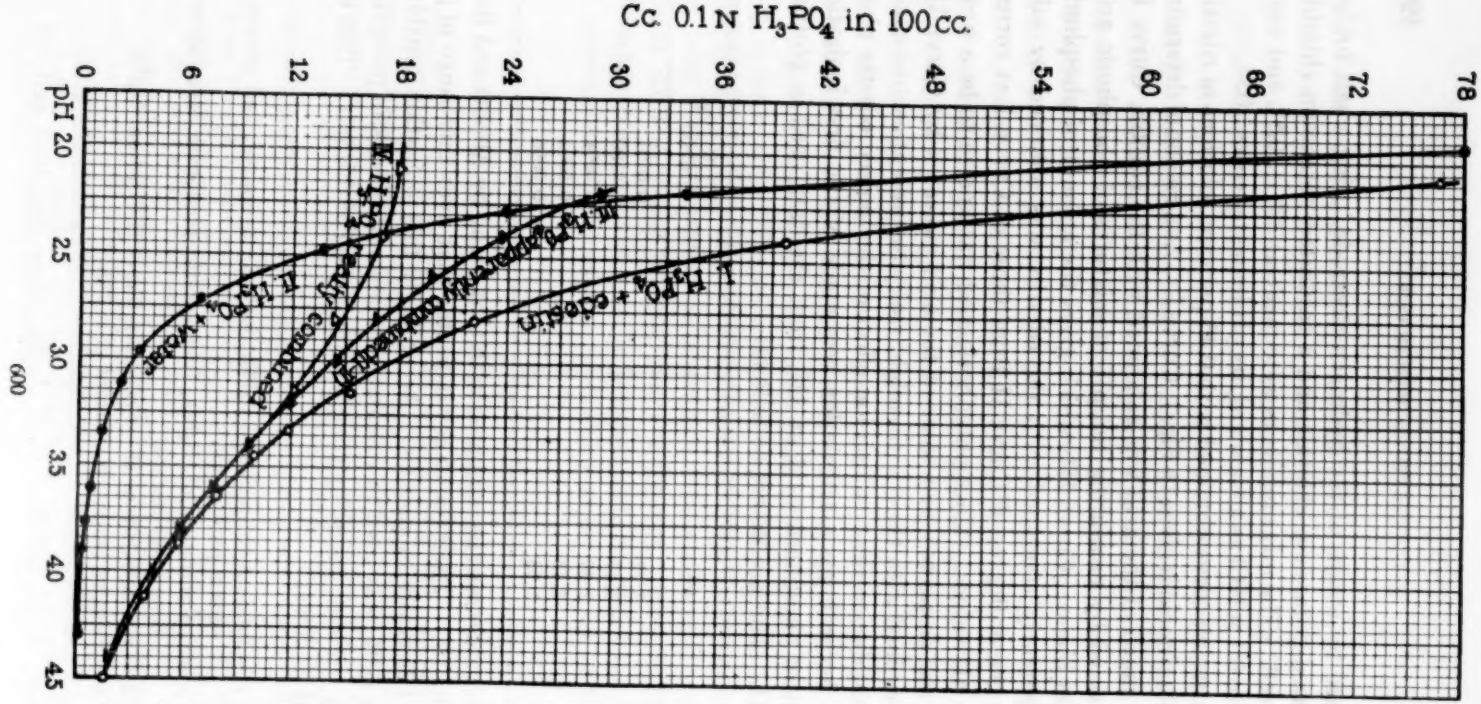


FIG. 1. I. Titration curve of 0.45 per cent edestin with 0.1 N H_3PO_4. II. Titration curve of water with 0.1 N H_3PO_4. III. Difference between I and II. IV. Amounts of H_3PO_4 combined with edestin, calculated from I and ionization constant of H_3PO_4.

Then

$$k = \frac{[H^+]\,[H_2PO_4^-]}{[H_3PO_4]} = \frac{h\,(h+x)}{c-h-x}$$

$$x = \frac{kc}{h+k} - h$$

The values of x obtained in this way, expressed in cc. of 0.1 N H_3PO_4 per 100 cc., are plotted in Curve IV, which indicates the amount of H_3PO_4 really combined with the edestin. It was found that above pH 3.4 the values so calculated did not differ much from those plotted in Curve III, although it is not strictly justifiable to consider H_3PO_4 as a monobasic acid in this range of pH, since the values obtained for k were not constant above pH 3.

Curves representing the amounts of hydrochloric, sulfuric, and oxalic acids combined with 0.45 gm. of edestin in 100 cc. are given in Fig. 2, along with the curve for H_3PO_4 as given in Fig. 1, Curve IV. In the case of these stronger acids the amount combined was obtained in the same way as Curve III, Fig. 1, by subtracting the ordinates of the acid-water curve from those of the acid-edestin curve. The curves for HCl and H_2SO_4 are nearly identical, indicating that each combines in equivalent proportions with edestin; *i.e.*, H_2SO_4 acts as a dibasic acid. The curve for $H_2C_2O_4$ above pH 4 is identical with with that for H_2SO_4, indicating that here oxalic acid is also dibasic. The curves soon diverge, however, and at the maximum that for oxalic acid is almost exactly twice as high as that for hydrochloric, indicating that here oxalic acid combines not in equivalent but in molecular proportions; *i.e.*, it is here a monobasic acid. The curve for H_3PO_4 seems to reach a maximum at a height a little over three times that of the curve for HCl and H_2SO_4, indicating that H_3PO_4 is combined with the edestin in molecular, not in equivalent proportions, or that H_3PO_4 combines with edestin as a monobasic acid.

In order to show how closely these proportions hold, and in what range of pH, the curves of Fig. 2 were plotted on a large scale and the values given in Table I were read off.

The values in Table I show that the ratios are most nearly as 1:2:3 in the region where the curves become horizontal, indicating that the edestin is all combined.

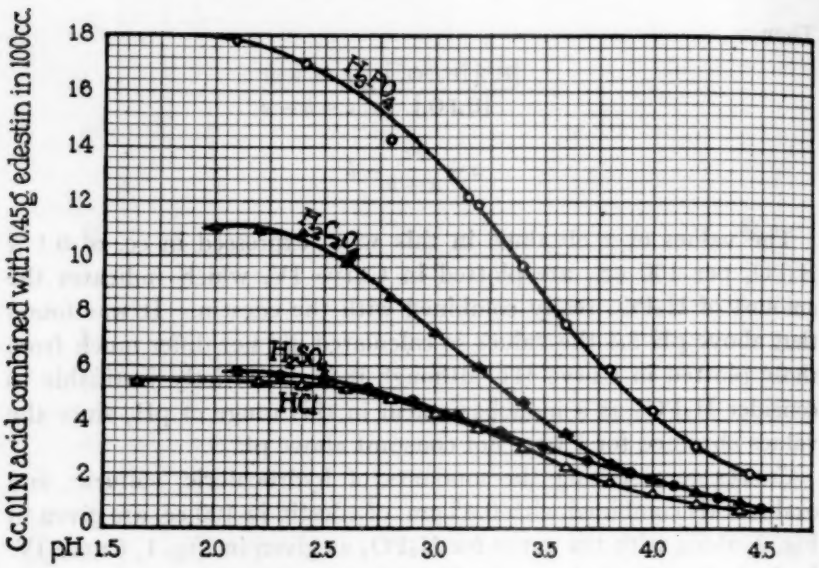


FIG. 2. Amounts of 0.1 N acid combined with 0.45 gm. edestin in 100 cc. Values for HCl, H_2SO_4, and $H_2C_2O_4$ obtained by difference between titration curves with and without protein. Values for H_3PO_4 obtained by calculation, as in Fig. 1, IV.

TABLE I.

Ratios of Amounts of Different Acids Combined with Edestin at Corresponding pH Values.

pH	HCl	H_2SO_4	$H_2C_2O_4$	H_3PO_4	Ratio $\frac{H_2SO_4}{HCl}$	Ratio $\frac{H_2C_2O_4}{HCl}$	Ratio $\frac{H_3PO_4}{HCl}$
	cc.	*cc.*	*cc.*	*cc.*			
2.1	5.6	5.8	10.8	17.8	1.0	1.9	3.2
2.4	5.3	5.6	10.8	17.0	1.1	2.0	3.2
2.7	5.05	5.2	9.15	15.4	1.0	1.8	3.0
3.0	4.3	4.4	7.2	13.0	1.0	1.7	3.0
3.3	3.35	3.6	5.35	10.1	1.1	1.6	3.0
3.6	2.3	2.8	3.5	6.95	1.2	1.5	3.0
3.9	1.5	2.05	2.1	4.2	1.3	1.4	2.8
4.2	0.95	1.35	1.3	2.1	1.4	1.4	2.2
4.5	0.6	0.75	0.85	0.8	1.3	1.4	1.3

Attempts were made to carry these curves farther into the region of lower pH, but since they are obtained from differences between the ordinates of steep curves like I and II in Fig. 1, the errors become too

great, as the experimental error in the pH determination is about ± 0.02 pH.

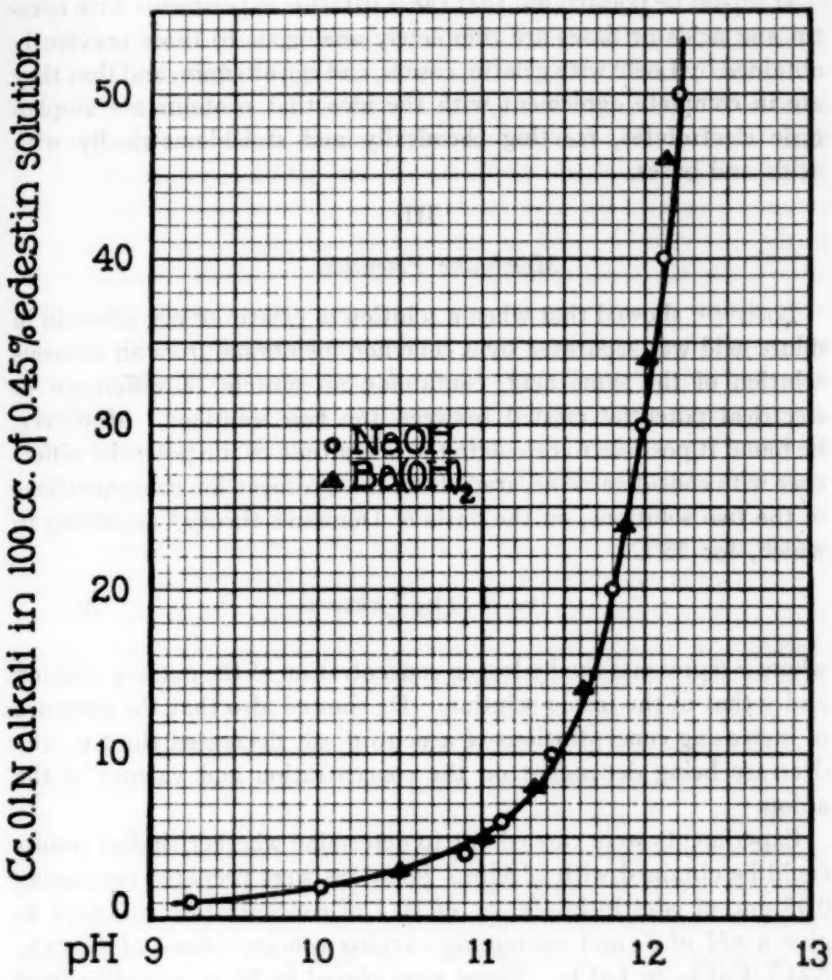


FIG. 3. Titration curve of 0.45 per cent edestin with 0.1 N NaOH and $Ba(OH)_2$.

Fig. 3 represents the titration of 0.45 per cent edestin solutions with 0.1 N NaOH and $Ba(OH)_2$. The curve is the original titration curve, and does not represent the amount of alkali combined with the edestin. However, since the points fall on one curve, it seems fairly

evident that the edestin must be combined with these strong alkalies in chemically equivalent proportions.

It should be pointed out that these titration experiments with edestin and acids or bases are completely analogous to those previously obtained by Loeb[1] with gelatin, casein, and egg albumin, and that they are in complete agreement with the idea that proteins are amphoteric electrolytes, reacting chemically and stoichiometrically with acids and bases.

III.

Membrane Potentials.

Loeb[8,9,10] showed that when a solution of gelatin or egg albumin in dilute acid was separated by a collodion membrane from an aqueous solution of the same acid, containing no protein, a difference in electrical potential existed between the two solutions. Moreover, he found it possible to calculate the magnitude of the potential difference with considerable accuracy from the hydrogen ion concentrations of the two solutions, on the basis of Donnan's theory,[2] according to which, for 25°C.,

$$\text{P.D.} = 59 \log \frac{x}{y} \text{ millivolts} \qquad (1)$$

where x represents the hydrogen concentration of the outside solution and y that of the inside solution. He showed also that the presence of increasing concentrations of a neutral salt decreased the P.D., the decrease being dependent on the concentration and valence of the anion.

Experiments were carried out to determine whether similar results could be obtained with edestin. Solutions were prepared containing 0.45 gm. of edestin in 100 cc. of HCl of concentration sufficient to give a pH of 3, and containing varying concentrations of Na_2SO_4, NaCl, $CaCl_2$, or $LaCl_3$. These were placed in 50 cc. collodion bags fitted with rubber stoppers and manometer tubes, and the bags were suspended in beakers of HCl of pH 3, which had been made up to

[8] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 557.
[9] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 667.
[10] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 351.

have the same salt concentration as the protein solutions. The beakers were set in a water thermostat at 25° ± 1°C. After equilibrium had been established (12 to 48 hours), the osmotic pressure was measured in terms of millimeters of the solution in the manometer tubes. The P.D. between the inside and outside solutions was determined in each case with the aid of saturated KCl calomel electrodes and a Compton electrometer. The P.D. measurements were made in a room at about 20°C., but the solutions were very nearly at 25°, since the P. D. was determined for each solution within 2 or 3 minutes after removing it from the thermostat at 25°. Finally the pH of the inside and outside solutions, at 33°, was determined with the hydrogen electrode and potentiometer. The calculated P. D. values were reduced to 25° by multiplying by $\frac{298}{306}$ the differences between the E.M.F. readings obtained for the two solutions with the hydrogen electrode. (This amounts to the same thing as using equation (1), but avoids slight arithmetical errors due to rounding off the values for pH.) The P.D. values were read to 0.5 millivolts, but the reproducibility was of the order of 1 millivolt.

The results of experiments with 0.45 per cent edestin chloride, at pH 3, and the four salts mentioned, are given in Tables II to V.

The excellent agreement of the observed and calculated values for the P.D. in these salt experiments proves that the Donnan equilibrium governs the effect of salt on the P.D. of edestin chloride solutions fully as well as in the case of gelatin or albumin chloride. Tables II and III show that here too it is the anion of the salt which has the depressing effect on the P.D., the sulfate ion being more effective than the chloride ion. An increase in the concentration of an ion of opposite charge to that of the protein ion tends to prevent the forcing of acid from the inside to the outside solution, and hence decreases the difference in pH and the resulting P.D. This may also be shown clearly from the results with the different chlorides by plotting the P.D. against the equivalent concentration of chloride ion furnished by the salt, assuming complete or equal ionization of the inorganic chlorides. The results in Tables III, IV, and V are so plotted in Fig. 4. It is evident that the effects of NaCl, $CaCl_2$, and $LaCl_3$ are identical, if compared at the same concentration of chloride ion. This proves beyond a doubt that the valence or nature of the cation is of no influence on the P.D. of edestin chloride.

TABLE II.

Effect of Sodium Sulfate on the P.H. *and Osmotic Pressure of 0.45 Per Cent Edestin Chloride at pH 3.*

Concentration of Na_2SO_4.	M/64	M/128	M/256	M/512	M/1,024	M/2,048	M/4,096	M/8,192	M/16,384	0
pH inside	Edestin precipitated.	2.93	2.86	2.83	2.88	2.90	2.93	2.97	2.99	3.00
pH outside		2.92	2.83	2.78	2.77	2.74	2.72	2.70	2.69	2.69
Observed P.D., *millivolts*		0.5	2.0	4.0	4.5	9.5	13.5	16.0	17.0	19.0
Calculated P.D., *millivolts*		0	1.5	3.5	7.0	9.5	12.5	15.5	17.0	18.0
Observed osmotic pressure, *mm*		6	10	24	48	71	106	144	172	204

TABLE III.

Effect of Sodium Chloride on the P.H. *and Osmotic Pressure of 0.45 Per Cent Edestin Chloride at pH 3.*

Concentration of NaCl.	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	0
pH inside	2.79	2.78	2.79	2.77	2.84	2.84	2.89	2.89	2.95	3.00
pH outside	2.79	2.77	2.77	2.74	2.77	2.74	2.75	2.70	2.72	2.70
Observed P.D., *millivolts*	0	0.5	1.0	2.0	3.0	6.5	8.0	10.5	14.0	17.5
Calculated P.D., *millivolts*	0	1.0	1.0	2.0	4.0	6.0	8.0	10.5	13.5	17.5
Observed osmotic pressure, *mm*	8	17	27	34	41	56	88	124	152	201

TABLE IV.

Effect of Calcium Chloride on the P.H. *and Osmotic Pressure of 0.45 Per Cent Edestin Chloride at pH 3.*

Concentration of $CaCl_2$.	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	M/2,048	M/4,096	0
pH inside	2.82	2.80	2.80	2.88	2.82	2.87	2.92	2.95	2.98	3.02
pH outside	2.82	2.78	2.78	2.78	2.75	2.75	2.72	2.70	2.72	2.70
Observed P.D., *millivolts*	0	0.5	1.5	3.0	5.0	8.0	11.0	14.0	15.5	15.5
Calculated P.D., *millivolts*	0	1.0	1.0	6.0	4.0	7.0	11.5	14.5	15.5	18.5
Observed osmotic pressure, *mm*	19	26	34	42	55	83	121	151	177	205

CORRECTION.

On page 606, Vol. iv, No. 5, in the italic headings for Tables II, III, and IV for P. H. read P. D.

J. gen. Physiol.

TABLE V.

Effect of Lanthanum Chloride on the P.D. *and Osmotic Pressure of 0.45 Per Cent Edestin Chloride at pH 3.*

Concentration of $LaCl_3$.	M/24	M/48	M/96	M/192	M/384	M/768	M/1,536	M/3,072	M/6,144	0
pH inside	2.79	2.79	2.80	2.79	2.80	2.85	2.87	2.95	2.98	3.00
pH outside	2.78	2.78	2.77	2.75	2.74	2.74	2.70	2.70	2.70	2.69
Observed P.D., *millivolts*	0	0.5	1.5	3.5	4.5	6.5	10.5	13.5	15.5	18.5
Calculated P.D., *millivolts*	0.5	0.5	2.0	2.5	4.0	7.0	10.0	14.5	16.5	18.5
Observed osmotic pressure, *mm*	20	26	33	41	54	82	117	154	180	199

TABLE VI.

Effect of pH on P.D. *and Osmotic Pressure of 1 Per Cent Edestin Chloride.*

pH inside	1.47	2.04	2.59	3.06	3.44	3.91	4.38	4.78	5.09
pH outside	1.43	1.91	2.32	2.61	2.90	3.23	3.61	3.91	4.15
Observed P.D., *millivolts*	5.0	7.5	14.0	23.0	29.0	39.0	42.5	37.0	32.5
Calculated P.D., *millivolts*	2.0	8.0	16.0	26.0	32.0	40.0	46.0	52.0	56.0
Observed osmotic pressure, *mm*	94	239	415	535	526	501	390	270	200

TABLE VII.

Effect of pH on P.D. *and Osmotic Pressure of 1 Per Cent Edestin Chloride in* M/512 *Sodium Chloride.*

pH inside	1.47	2.02	2.51	2.92	3.30	3.69	4.20	4.73	5.09
pH outside	1.43	1.90	2.29	2.62	2.98	3.38	3.94	4.48	4.81
Observed P. D., *millivolts*	2.0	7.5	12.5	17.0	18.0	16.5	12.5	8.0	4.5
Calculated P. D., *millivolts*	2.0	7.5	12.5	17.5	18.5	18.5	15.5	14.5	16.5
Observed osmotic pressure, *mm*	82	191	328	383	331	246	134	65	28

TABLE VIII.

Effect of pH on P.D. *and Osmotic Pressure of 1 Per Cent Edestin Acetate in* M/100 *Sodium Acetate.*

pH inside	3.25	3.45	3.57	3.74	3.94	4.10	4.21	4.39	4.51	4.67
pH outside	3.12	3.35	3.48	3.64	3.86	4.04	4.15	4.34	4.46	4.62
Observed P.D., *millivolts*	6.5	6.0	5.5	5.0	4.5	4.5	3.5	3.0	2.5	2.5
Calculated P.D., *millivolts*	7.5	6.0	5.5	6.5	4.5	3.5	3.5	3.0	3.0	2.5
Observed osmotic pressure, *mm*	139	114	102	88	71	62	56	49	44	38

The effect of the hydrogen ion concentration on the P.D. was determined by using 1 per cent solutions of edestin in varying concentrations of HCl without the addition of salt. The results of such an experiment are given in Table VI and Fig. 5.

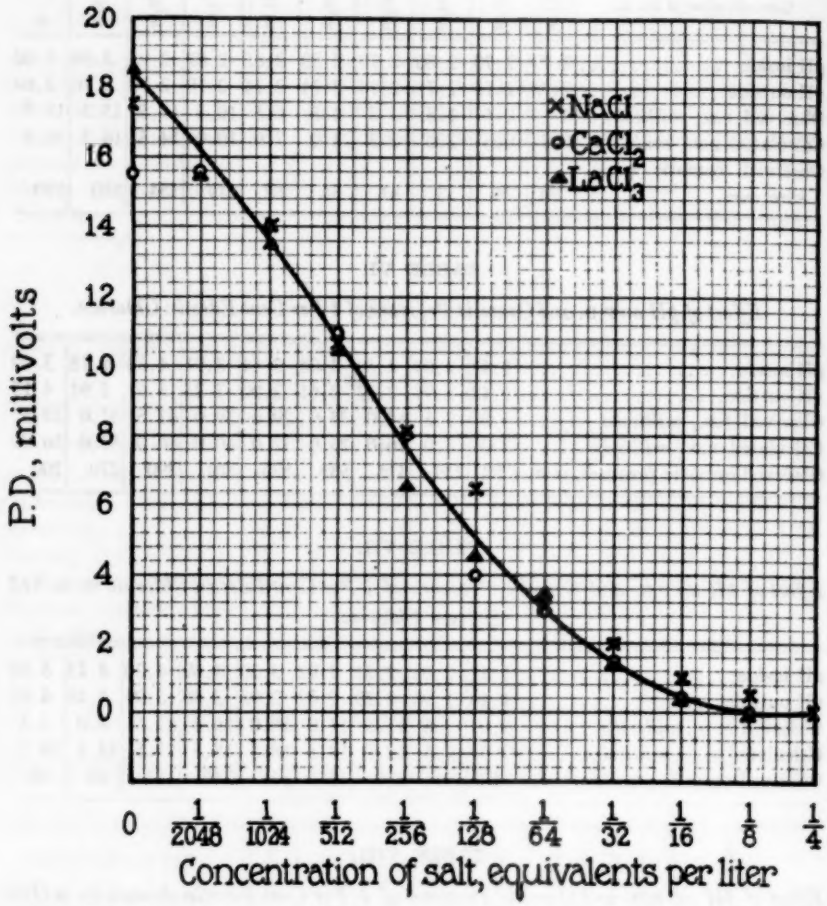


FIG. 4. Effect of different chlorides on P.D. observed with 0.45 per cent edestin chloride at pH 3.

It will be noticed that the observed and calculated values for P.D. agree fairly well up to pH 4.0. The results therefore show that Donnan's theory does apply quantitatively to edestin chloride solutions between pH 1.5 and 4.0.

Since it had been noticed that the agreement between observed and calculated P.D. was better when the solution had a higher concentration of electrolyte, the experiment of Table VI was repeated with all solutions M/512 with respect to NaCl. The results are given in Table VII.

The agreement with the theory is excellent up to pH 3, but again becomes poor above pH 4. In comparing Tables VI and VII it may be observed that the presence of M/512 NaCl had more effect in decreasing the P.D. of those solutions which contained less HCl. This

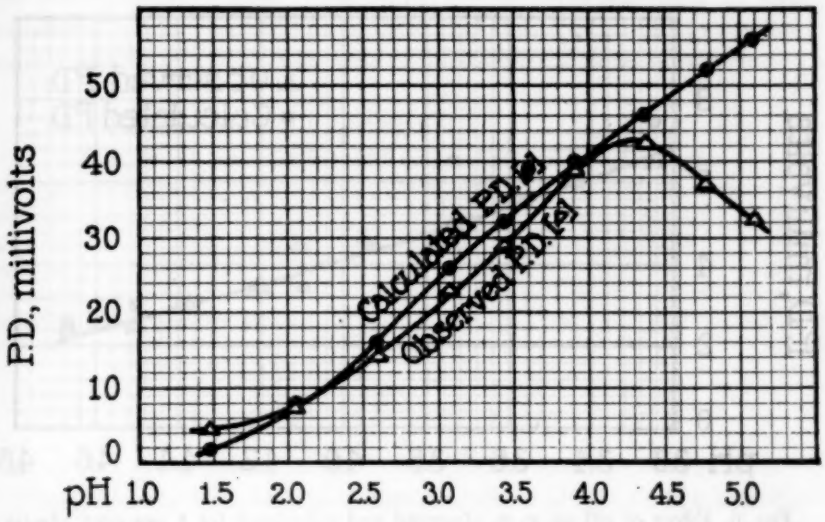


FIG. 5. Effect of pH on P.D. observed and calculated for 1 per cent edestin chloride.

is in line with the experiments already described on the effect of salt concentration on the P.D., indicating that the depression of the P.D. is due to the anion.

In order to obviate any error in the calculated P.D. which might be due to difficulty in determining accurately the pH of the very dilute acid in some of the outside solutions, an experiment was run in which both inside and outside solutions were buffered by M/100 sodium acetate and different concentrations of acetic acid. The results are given in Table VIII and Fig. 6.

It will be observed that the agreement between the observed and calculated values for the P.D. is within the experimental error, and that the experiment includes that range of pH where the values did not agree well in the case of the solutions without buffer. The curve in Fig. 6 is of a different shape from that in Fig. 5 because the depressing action of the anion of the salt has more effect in the less acid solutions, since the concentration of the anion furnished by the acid is originally less in such solutions. The existence of a difference in pH in such buffered solutions and the agreement between the observed

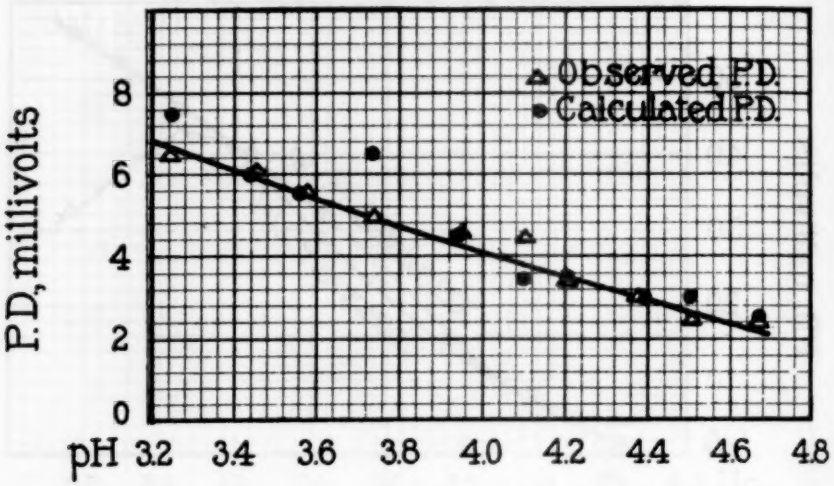


FIG. 6. Effect of pH on P. D. observed and calculated for 1 per cent edestin acetate in M/100 sodium acetate.

and calculated values for the P.D. constitute a striking proof of the validity of Donnan's theory. This experiment, therefore, supplements the results in Tables VI and VII by proving that Donnan's theory applies to solutions of edestin in acid in the region near the isoelectric point as well as in more acid solutions.

A further consequence of Donnan's theory, as applied by Loeb to amphoteric colloids, is that the pH inside should become less than the pH outside on the alkaline side of the isoelectric point of the protein; *i.e.*, the P.D. should be opposite in sign to that found on the acid side. This was tested by working with solutions of edestin in NaOH. It

was found that the solubility of edestin in acid or alkali was less than 0.45 gm. in 100 cc. for a broad zone around the isoelectric point, from about pH 5 to pH 9. Table IX gives the results of a few measurements that were made with 0.45 per cent sodium edestinate in sodium hydroxide. Both inside and outside solutions were protected from the CO_2 of the air by soda-lime tubes.

These results show that the prediction of the theory was confirmed, for the pH outside is now greater than the pH inside, and the sign of both observed and calculated P.D. is opposite to that obtained with the acid solutions. Moreover, the agreement between observed and calculated P.D. is close enough to show that the Donnan theory applies quantitatively on the alkaline side of the isoelectric point of edestin.

TABLE IX.

P.D. and Osmotic Pressure of 0.45 Per Cent Sodium Edestinate.

pH inside	9.93	10.41	10.96
pH outside	10.12	10.55	11.05
Observed P.D., *millivolts*	−9.5	−8.5	−6.0
Calculated P.D., *millivolts*	−11.0	−8.0	−6.0
Observed osmotic pressure, *mm*	28	37	45

IV.

Osmotic Pressure.

The similarity between the depressing effect of salt on the osmotic pressure and on the P.D. in the case of gelatin chloride has been pointed out by Loeb.[9] That the same resemblance exists in the case of edestin chloride is shown by the results in Tables II to V, and by a comparison of Fig. 7 with Fig. 4. Since the abscissæ in Fig. 7 represent equivalent concentrations of salt with respect to chloride ion, and the points obtained with the three chlorides all fall on the same curve, it is evident that it is again only the anion that is effective in depressing the osmotic pressure of a solution in which the protein ion is positive. The nature or valence of the cation seems to have no effect. These results are in contradiction to the theory that the effect of a salt on such colloidal properties of protein solutions is due to the adsorption of both ions of the salt, but are in complete accord with the results of

Loeb on gelatin. The true explanation of the effect of salts on these properties of protein solutions is found in Donnan's theory of membrane equilibrium.

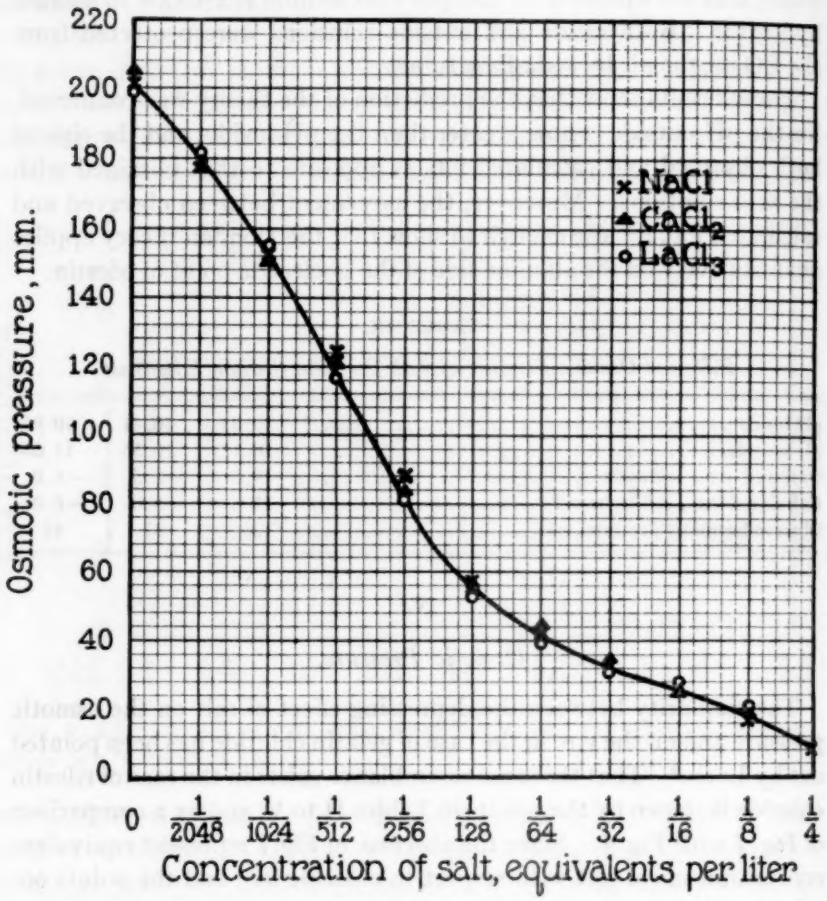


FIG. 7. Effect of different chlorides on osmotic pressure observed with 0.45 per cent edestin chloride at pH 3.

In studying the effect of hydrogen ion concentration on the osmotic pressure of gelatin chloride, Loeb[9] found that the curves obtained were of the same general shape as the P.D. curves, but that the maximum for osmotic pressure was at a slightly lower pH. He later[10]

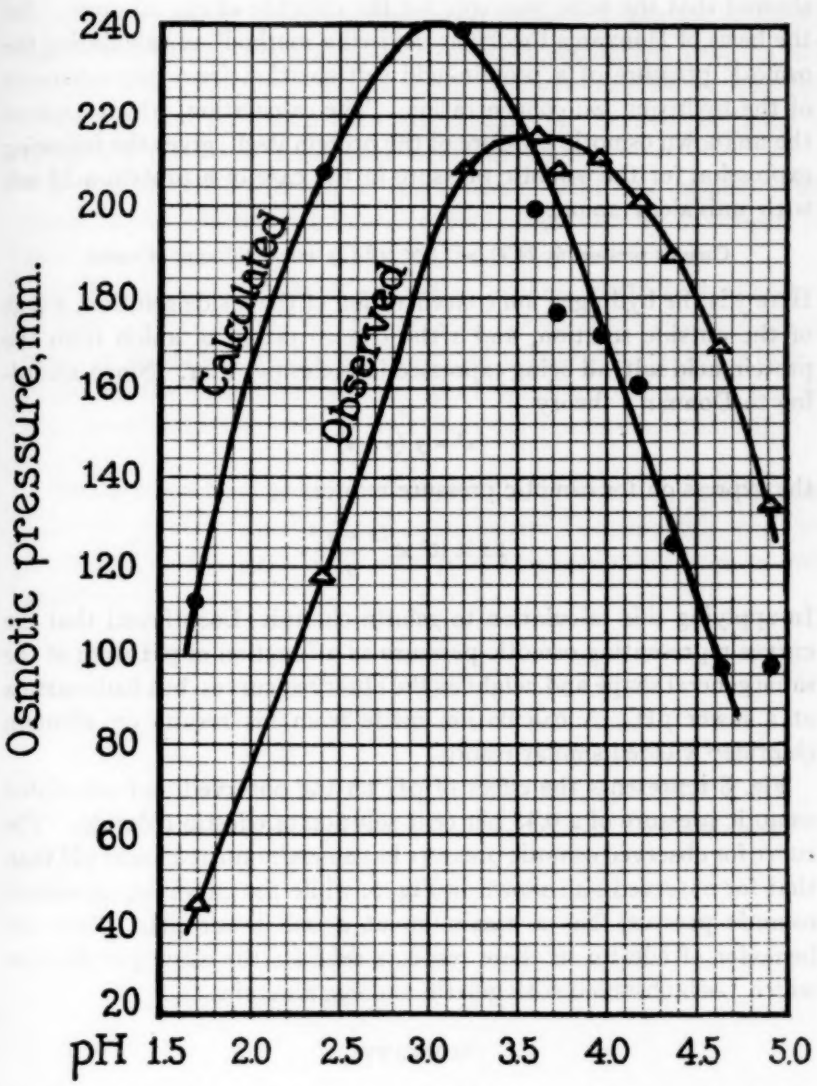


FIG. 8. Effect of pH on observed and calculated osmotic pressure of 0.45 per cent edestin chloride.

showed that the same was true for the chloride of egg albumin. On the basis of Donnan's theory he devised a method[11] of calculating the osmotic pressure of a protein-acid salt solution from measurements of the hydrogen ion concentration. The calculation, which neglects the unknown osmotic pressure of the protein itself, gives the following expression for the osmotic pressure in the case of a protein-acid salt with univalent anion:

$$\text{Osmotic pressure at } 24^\circ\text{C.} = 2.5 \times 10^5 \ (2y + z - 2x) \text{ mm. of water.}$$

Here y is the hydrogen ion concentration of the inside solution, x that of the outside solution, and z the concentration of anion from the protein-acid salt, all being expressed in moles per liter. Since according to Donnan's theory

$$x^2 = y \ (y + z)$$

the expression for osmotic pressure reduces to

$$2.5 \times 10^5 \times \frac{(x - y)^2}{y}$$

In applying this calculation to gelatin chloride, Loeb found that the curves representing osmotic pressure as a function of pH were of the same general shape and height as the observed curves, but had maxima at a lower pH. A calculation made from his results on albumin chloride[10] yielded similar results.

Fig. 8 represents the effect of pH on the observed and calculated osmotic pressure of a 0.45 per cent solution of edestin chloride. The curve for observed osmotic pressure has a maximum at a lower pH than that for observed P.D. shown in Fig. 5, while the curve for calculated osmotic pressure has a maximum at a still lower pH. Thus the behavior of edestin in these respects exhibits the same peculiarities which Loeb observed with gelatin and egg albumin.

SUMMARY.

1. It has been shown by titration experiments that the globulin edestin behaves like an amphoteric electrolyte, reacting stoichiometrically with acids and bases.

[11] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 691.

2. The potential difference developed between a solution of edestin chloride or acetate separated by a collodion membrane from an acid solution free from protein was found to be influenced by salt concentration and hydrogen ion concentration in the way predicted by Donnan's theory of membrane equilibrium.

3. The osmotic pressure of such edestin-acid salt solutions was found to be influenced by salt concentration and by hydrogen ion concentration in the same way as is the potential difference.

4. The colloidal behavior of edestin is thus completely analogous to that observed by Loeb with gelatin, casein, and egg albumin, and may be explained by Loeb's theory of colloidal behavior, which is based on the idea that proteins react stoichiometrically as amphoteric electrolytes and on Donnan's theory of membrane equilibrium.

The writer wishes to acknowledge his indebtedness to Dr. Jacques Loeb, at whose suggestion and under whose direction this work was done, and to Dr. John H. Northrop, whose advice has been of great assistance.

THE ELIMINATION OF DISCREPANCIES BETWEEN OBSERVED AND CALCULATED P.D. OF PROTEIN SOLUTIONS NEAR THE ISOELECTRIC POINT WITH THE AID OF BUFFER SOLUTIONS.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 30, 1922.)

The writer has shown in a series of papers[1] that the P.D. observed between a solution of gelatin chloride or albumin chloride inside a collodion bag and an outside solution free from gelatin could be calculated from the difference in the hydrogen ion concentration between the inside and outside solutions. The agreement between the observed and calculated values was perfect when the solution contained a neutral salt or when the hydrogen ion concentration of the solution was not too close to that of the isoelectric point; the agreement was, however, less satisfactory when the pH was near that of the isoelectric point of gelatin, *i.e.*, near pH 4.7, and no salts were present.[2] The source of this disagreement seemed to lie in the inaccuracy in the measurement of the pH of the aqueous solution free from gelatin (the outside solution) at a pH between 4.0 and 7.0. If this surmise was correct, the disagreement in that region of hydrogen ion concentrations should be caused to disappear by the use of a buffer solution inside and outside.

1 per cent solutions of isoelectric gelatin were made up in M/100 Na acetate solutions containing varying amounts of 1 M acetic acid so that the pH of the gelatin solution varied (at the end of the experiment) between 4.65 (*i.e.*, practically isoelectric gelatin) and 3.34 (Table I.) Collodion bags, of a content of about 50 cc., were filled

[1] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 667; 1921–22, iv, 351.

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 138, 156.

with these solutions of gelatin in buffer solutions as described in previous papers. The bags were put into beakers containing 350 cc. of identical solutions of M/100 Na acetate and 1 M acetic acid as those inside the bags, except that the 350 cc. outside solutions contained no gelatin. The temperature was 24°C. After 24 hours the osmotic pressure, the P.D. between inside and outside solutions, and the pH of the inside and outside solutions were measured. From the value pH inside minus pH outside the P.D. was calculated, and Table I shows that the P.D. thus calculated agrees with the observed P.D. The rest of the table needs no explanation.

TABLE I.

Influence of pH on P.D. *of Solutions of Gelatin Acetate in the Presence of Buffer Solution.*

Cc. 1 M acetic acid in 100 cc. inside and outside solutions.	1.0	1.5	2.0	3.0	4.0	6.0	10.0	15.0	20.0	30.0
Osmotic pressure, in mm. H_2O	21	31	34	43	47	62	83	95	103	108
pH inside	4.65	4.52	4.40	4.23	4.14	3.99	3.76	3.61	3.49	3.34
pH outside	4.65	4.50	4.37	4.19	4.09	3.92	3.69	3.53	3.39	3.23
pH inside minus pH outside	0	0.02	0.03	0.04	0.05	0.07	0.07	0.08	0.10	0.11
P.D. calculated, *millivolts*	0	1.0	2.0	2.5	3.0	4.0	4.0	5.0	5.5	7.0
P.D. observed, *millivolts*	0.5	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.5	6.0

Similar results were obtained, in the case of solutions of edestin, by Dr. Hitchcock, whose paper appears simultaneously with this note.[3]

SUMMARY AND CONCLUSIONS.

1. It had been noticed in the previous experiments on the influence of the hydrogen ion concentration on the P.D. between protein solutions inside a collodion bag and aqueous solutions free from protein that the agreement between the observed values and the values calculated on the basis of Donnan's theory was not satisfactory near the

[3] Hitchcock, D. I., *J. Gen. Physiol.*, 1921–22, iv, 597.

isoelectric point of the protein solution. It was suspected that this was due to the uncertainty in the measurements of the pH of the outside aqueous solution near the isoelectric point. This turned out to be correct, since it is shown in this paper that the discrepancy disappears when both the inside and outside solutions contain a buffer salt.

2. This removes the last discrepancy between the observed P.D. and the P. D. calculated on the basis of Donnan's theory of P.D. between membrane equilibria, so that we can state that the P.D. between protein solutions inside collodion bags and outside aqueous solutions free from protein can be calculated from differences in the hydrogen ion concentration on the opposite sides of the membrane, in agreement with Donnan's formula.

ELECTRICAL CHARGES OF COLLOIDAL PARTICLES AND ANOMALOUS OSMOSIS.

II. Influence of the Radius of the Ion.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 21, 1922.)

The valency and sign of charge of ions are not the only variables influencing anomalous osmosis. A third variable is the radius of the ion. The radius of the ions of the alkali metals increases in the order of Li < Na < K. The writer has shown that when solutions of pH 3.0 of the chlorides or nitrates of these cations are separated from a solution of water also of pH 3.0 by collodion-gelatin membranes, water diffuses into the salt solution with a rate increasing inversely with the radius of the cation.[1] This is illustrated in the transport curves in Fig. 1 where the abscissæ are the concentration of the salt and the ordinates the number of millimeters to which the level of liquid has risen in 20 minutes in the manometer connected with the solution. It is obvious that the rise is greatest for LiCl, less for NaCl, and still less for KCl. This confirms the results of a preceding publication.

It is also obvious that the three transport curves in Fig. 1 show the initial rise to a maximum at about M/16 followed by a drop which is followed by a second rise. This second rise will not interest us here since it is mainly or exclusively the expression of the transport of liquid due to osmotic forces.[2] Only part of the curves, namely, between the concentration from 0 to a concentration of M/4, is due to electrical forces, and only these forces interest us in this connection.

The question arises, What determines this influence of the radius of the monatomic and monovalent cations on the electrical transport

[1] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 673.

[2] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 173; 1921–22, iv, 463.

of water? The idea prevalent in work on cataphoresis, electrical endosmose, or current potentials seems to be that the charge of the particle or membrane is due to the adsorption of the ions of the salt. Thus the transport curves in Fig. 1 would suggest that the cations

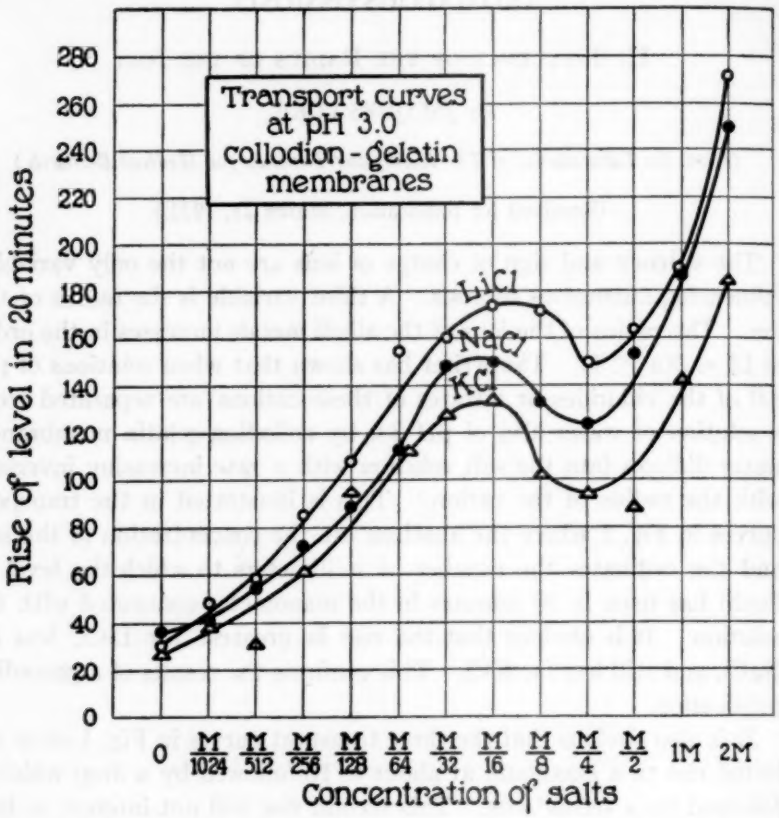


FIG. 1. Influence of Li, Na, and K on rate of electrical transport of liquid through a collodion-gelatin membrane at pH 3.0. The rate of transport increases inversely with the radius of the cation.

increase the positive charge of the walls of the pores in the gelatin membrane and that this increase occurs inversely with the radius of the three cations.

The influence of the three cations on the charge of gelatin particles was measured directly in the following way. Doses of 1 gm. of pow-

dered gelatin particles of a definite size (going through a sieve with mesh No. 30 but not through mesh No. 60) were rendered isoelectric and then put into 200 cc. of various concentrations of KCl, LiCl, or NaCl, made up in water containing 16 cc. of 0.1 N HCl at 20°C. After 2 hours, during which the mixtures were stirred frequently, the gelatin was separated from the supernatant liquid by filtration, and after this the gelatin was melted and poured into special glass vessels,

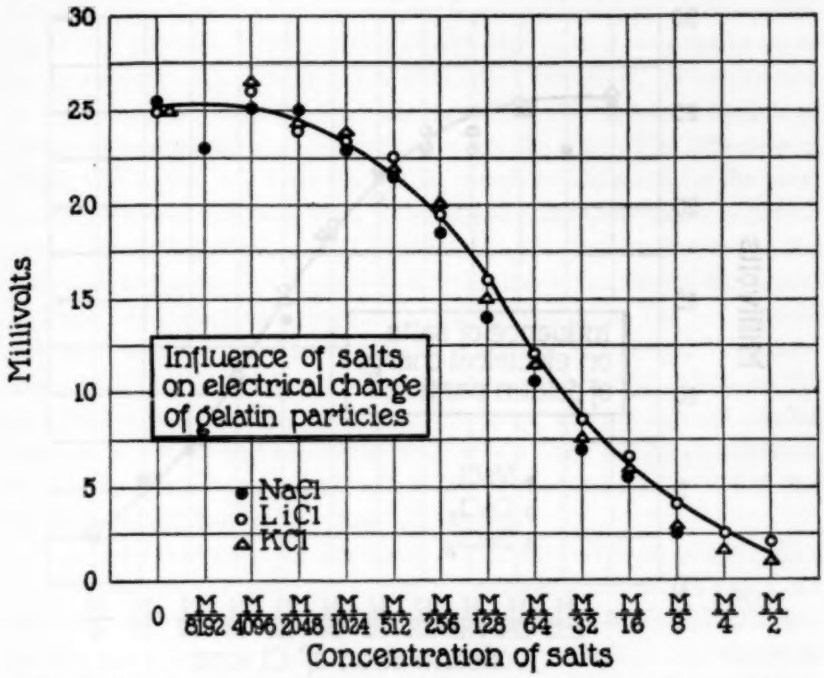


FIG. 2. Influence of LiCl, NaCl, and KCl on P.D. between solid particles of gelatin chloride and surrounding liquid at pH 3.0. This influence is the same for all three salts, suggesting that there exists only a depressing influence of the anion but no opposite influence of the cation on this P.D. Abscissæ are the concentration of the salts; ordinates, observed P.D.

and allowed to solidify in the vessels standing on ice for 1 hour. The supernatant solution was also cooled in the same way. The P.D. between the solid gelatin and the supernatant liquid with which it was in equilibrium was then measured at a temperature of about 5°C. with calomel electrodes and saturated solutions of KCl by a Compton

electrometer. The details of the procedure can be found in a book which is about to appear.[3]

Fig. 2 shows that the three salts, KCl, NaCl, and LiCl depress the P.D. between solid gelatin chloride and the liquid with which the gelatin is in equilibrium in exactly the same way, since the values expressing the effect of the three salts on the P.D. lie on the same curve (Fig. 2). The ordinates of these curves in Fig. 2 are the observed

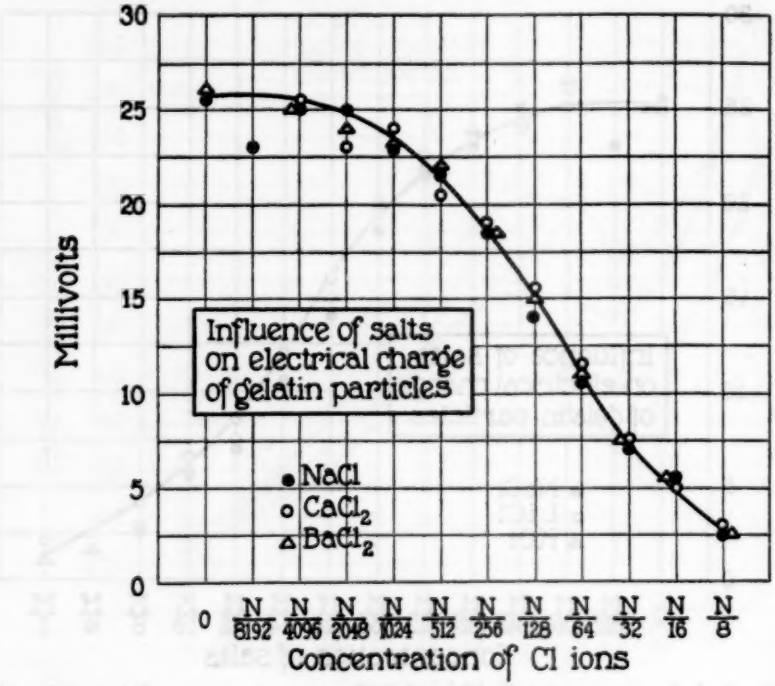


FIG. 3. Influence of NaCl, $CaCl_2$, and $BaCl_2$ on P.D. between solid granules of gelatin chloride and surrounding liquid at pH 3.0. Abscissæ are the concentration of Cl; ordinates, observed P.D. The influence is the same for the three salts, proving that there exists only a depressing effect of the Cl ion but no opposite effect of the cation.

P.D. and the abscissæ the concentrations of the salt. These curves contradict the idea that the three cations, Li, Na, and K influence the P.D. between gelatin and water by adsorption, and, moreover, they

[3] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

contradict the idea that the P.D. increases inversely with the radius of the three cations.

It has already been stated in the preceding paper[4] that at pH 3.0 only the anion of the salt has any effect on the P.D between gelatin chloride and the liquid with which it is in contact and that this effect is only depressing. The cation of the salt has no effect. This is shown in Fig. 3, where the influence of NaCl, $CaCl_2$, and $BaCl_2$ on the P.D. between solid gelatin chloride and the liquid with which it is in equilibrium are plotted. The method of the experiments was the same as in the experiments with LiCl and NaCl just described. The abscissæ in Fig. 3 are the chlorine ion concentrations (on the assumption of complete ionization) and the ordinates are the P.D. The influence of NaCl, $CaCl_2$, and $BaCl_2$ on the P.D. is, therefore, the same for the same concentration of chlorine ions, which means that there exists at pH 3.0 only a depressing effect of the Cl ions on the P.D., but no increase of the P.D. through adsorption of cations. If this latter effect existed the curves in Figs. 2 and 3 representing the influence of salts on the P.D. should not be identical. The identity of the curves in Figs. 2 and 3 can only mean that that ion of a salt which has the same sign of charge as the protein ion has no effect on the P.D. between the particles of gelatin chloride of pH 3.0 and the liquid with which they are in equilibrium. It may be stated incidentally that this was to be expected if the Donnan equilibrium is the cause of the P.D.

If we now return to the interpretation of the influence of the radius of the cation on the transport curves in anomalous osmosis in Fig. 1, we must infer that the difference in the transport curves for KCl, NaCl, and LiCl is not due to any influence of the three cations on the electrical double layer inside the pores of the membrane. We have seen in the preceding paper that there exists a second P.D. which has an influence on the transport curves, namely, the P.D. across the membrane, which is essentially but not exclusively due to a difference in the mobility of the oppositely charged ions. This P.D. was measured at the beginning of each transport experiment and at the end, *i.e.*, after 20 minutes. By that time some of the salt had diffused from the salt solution into the surrounding water. Table I gives the P.D. across the membrane

[4] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 463.

at the beginning and Table II gives it at the end of the experiment. The salt solution was positively charged and the outside solution was negatively charged. The reader will notice that as soon as the concentration is above M/64 the P.D. across the membrane increases in the order of KCl < NaCl < LiCl, and this corresponds to the difference in the transport curves in Fig. 1 which also begins to become

TABLE I.

Influence of Concentration of Salt on the Value of E.

P.D. in millivolts across a collodion-gelatin membrane between different concentrations of salts of pH 3.0 against H_2O of pH 3.0 (acid used, HCl), *at beginning of experiment.* Sign of charge of salt solutions positive.

Concentration.	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M	2 M
	millivolts	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*
KCl.....	5.0	5.0	6.5	7.5	8.0	7.5	7.5	5.5	4.5	3.5	3.5	3.0
NaCl.....	8.0	12.0	13.0	18.0	22.0	20.0	23.0	23.0	24.0	25.0	28.0	28.0
LiCl.....	6.5	9.0	12.5	18.0	21.0	25.5	27.0	35.0	37.0	37.0	40.0	42.5

TABLE II.

Influence of Concentration of Salt on the Value of E.

P.D. in millivolts across a collodion-gelatin membrane between different concentrations of salts of pH 3.0 against H_2O of pH 3.0 (acid used, HCl), *after 20 minutes from beginning of experiment.* Sign of charge of salt solutions positive.

Concentration.	M/1,024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M	2 M
	millivolts	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*
KCl.....	4.0	4.0	6.0	6.0	6.5	6.0	5.0	3.5	2.5	1.5	1.5	0.5
NaCl.....	7.5	12.0	10.0	13.0	16.5	16.0	16.0	15.0	11.0	12.0	10.5	.8.0
LiCl.....	6.5	10.0	13.0	18.0	19.0	24.0	22.0	25.0	22.0	19.5	17.0	15.0

marked when the concentration of the salts rises above M/64. We must, therefore, conclude that the difference in the transport curves in Fig. 1 is due to the difference in the influence of KCl, NaCl, and LiCl on the P.D. across the membrane. This P.D. is, perhaps, essentially, but not exclusively, a diffusion potential. Since K has the greatest and Li the smallest mobility of the three ions, it is to be expected that the diffusion potentials lead to such differences in the

P.D. as are expressed in Tables I and II. This fact has already been discussed, but it may be necessary to return to it in a later publication.

SUMMARY AND CONCLUSIONS.

1. When solutions of KCl, NaCl, or LiCl are separated from water without salt by a collodion-gelatin membrane and when the pH of both salt solution and water are on the acid side of the isoelectric point of gelatin, water diffuses from the side of pure water into the salt solution at a rate increasing inversely with the radius of the cations.

2. The adsorption theory would lead us to assume that this influence of the cations is due to an increase of the P.D. between the liquid and the membrane inside the pores of the gelatin film of the membrane, but direct measurements of this P.D. contradict such an assumption, since they show that the influence of the three salts on this P.D. is identical at pH 3.0.

3. It is found, however, that the P.D. across the membrane is affected in a similar way by the three cations as is the transport of water through the membrane.

4. This P.D. across the membrane varies inversely as the relative mobility of the three cations which suggests that the influence of the three cations on the diffusion of liquid through the membrane is partly if not essentially due to a diffusion potential.